Improving Electrochemical Techniques for Studying Dissimilatory Metal Reducing Bacteria

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Komal Joshi

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Advisor: Daniel R. Bond, PhD

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Chapter 1

Introduction

Advancements in the field of biotechnology in the past few decades have provided solutions to many research problems and have paved the way to new scientific discoveries. From development of different applications of microbial fuel cells and discovery and characterization of new classes of microorganisms to the progress in sequencing and annotation methods, the world of biotechnology is expanding every day. Currently, the world is dependent on fossil fuels to meet its energy demand but for future, we have to come up with other strategies to fulfill global energy needs.

One strategy is the use of **living fuels** where microbes are engineered to produce compounds of interest. The dissimilatory metal reducing bacteria utilize metals including Fe(III), Mn(IV), and U(VI) as terminal electron acceptors for anaerobic respiration. This process known as **dissimilatory metal reduction** is used by microbes to conserve energy by oxidizing organic electron donor and reducing metals. These bacteria possess the ability to transfer electrons outside the cell and in turn reduce the insoluble metal. This type of metabolism has practical applications for cleaning up environments contaminated with toxic metals.

This ability of **extracellular electron transfer** can be applied to many biotechnological applications. An area of focus for the application of extracellular electron transfer is bioremediation by using these bacteria in microbial fuel cells for wastewater treatment where metals are replaced by an electrode, which serves as the electron sink. The bacteria oxidizes organic matter present in the wastewater to carbon

dioxide and smaller carbon compounds which can be utilized by other species of bacteria and completely oxidized to carbon dioxide resulting in clean water and no other byproducts.

For living fuel technology to be used practically, three major challenge areas must be addressed: scalability, control over operation process, and defined biological pathways. First, anaerobic bioelectrochemical systems must be scalable from the laboratory to an industrial setting to make this technology feasible over large scales. Research is required for designing systems where parameters such as oxygen concentration, temperature, gas flow rate, energy recovery and storage, and evaporation rates can be simultaneously controlled. Since, this concept of utilizing living fuels is fairly new, a lot of study to understand the mechanism of electron transfer to extracellular electron acceptors as well as mechanism for interspecies electron transfer is required. We should also be able to engineer metabolic systems in these bacteria for desired products to be able to use this technology practically in an economically viable setting.

In the studies that follow, I have focused on aspects related to increasing control of reactors and further defining biological pathways in an organism capable of extracellular electron transfer, *Shewanella oneidensis* strain MR-1. *S. oneidensis* is a well-studied gram-negative gamma proteobacterium whose genome sequence is known and is easy to engineer. In chapter 2, work was done to improve the coulombic efficiency of *S. oneidensis* in reactors by improving design of anoxic bioreactors. The improved system can be used for studying phenotypes of various mutants of *S. oneidensis* as well as other metal reducing bacteria under anaerobic conditions on electrodes in order to

understand the process of extracellular electron transfer by studying the components involved in the process. In chapter 3, we monitored hydrogen metabolism in *S. oneidensis* biofilms and quantified the role of hydrogenases in electron transfer from *S. oneidensis* to electrodes in a bioreactor. In chapter 4, I sequenced the versions of pGUT2 and pGUT2PET plasmid to understand the adaptive evolution strategies of *S. oneidensis* for faster growth and utilization of the non-native substrate, glycerol and its subsequent conversion to acetate. Chapter 4 also includes differences in reduction rates of different electron acceptors in presence of glycerol as the sole electron donor.

Chapter 2

Modification of design of electrochemical cells for improving the coulombic efficiency of bioreactors for *Shewanella oneidensis*

Introduction

Life is dependent on transfer of electrons and redox balance. The energy currency of a cell is ATP. When an electron donor is oxidized, electrons are transferred via different multi-heme cytochromes to reduce a terminal electron acceptor and generate a proton gradient which can be used to make ATP. Under aerobic conditions, oxygen serves as the terminal electron acceptor. Aerobic respiration yields high biomass and metabolic rates because the potential difference between the electron donor (carbon source) and oxygen (electron acceptor) is high enough to support fast growth rates and biomass production. Under anaerobic respiration conditions, compounds other than oxygen (fumarate, nitrate, sulfate, metals such as Fe(III), Mn(IV), and poised electrodes) serve as terminal electron acceptors. All these compounds have lower redox potential than oxygen which results in slower growth rates. Most of the metal electron acceptors are insoluble compounds.

With the discovery of metal reducing bacteria capable of respiring electrodes (Bond et al., 2002, Gregory et al., 2004, Wang et al, 2008) the techniques of electrochemistry are now used to study microbial metabolism in dissimilatory metal reducing bacteria. **Bioelectrochemical systems** use electrochemically active bacteria to oxidize organic electron donors and reduce electrodes, which are a proxy to insoluble metals (Kim et al., 2002, Bond and Lovley, 2003, Logan, 2009). One of the very well

applied example of bioelectrochemical systems is the microbial fuel fell used in power generation and wastewater treatment research. Other examples of application of bioelectrochemical systems include remediation of sites contaminated with heavy toxic metals.

Shewanella oneidensis MR-1 is a model organism for studying microbial metal reduction. *S. oneidensis* is widely used in fuel cell studies due to the ease of culturing and its repertoire of respiration strategies. *S. oneidensis* MR-1 is a facultative anaerobic bacterium which possess the ability to respire a diverse range of electron acceptors (fumarate, dimethyl sulfoxide, trimethylamine oxide, sulphur, Fe(III), Mn(IV), U(VI) etc. under anaerobic conditions and oxygen under aerobic conditions). This ability is central to the applications of *S. oneidensis* in bioremediation and wastewater treatment systems (Bretschger et al, 2007, Carpentier et al., 2003). However, *S. oneidensis* has a poor current generation ability compared to some members in the *Geobacteraceae* family (Bond and Lovley, 2003, Bretschger et al, 2007).

Previous studies utilizing *S. oneidensis* MR-1 in bioelectrochemical systems were performed under insufficient anaerobic conditions resulting to loss of electrons to oxygen resulting in low coulombic efficiency. Coulombic efficiency is the ratio of amount to electrons transferred to electrodes to the amount of substrate oxidized (Bond and Lovley, 2003, Logan et al, 2006). Oxygen in these bioreactors resulted in planktonic growth when studying *S. oneidensis* using poised electrodes as the sole electron acceptor. Extracellular electron transfer in *S. oneidensis* takes place either by direct electron transfer to electrodes, electrons mediated through electron shuttles like flavins or both (Marsili et

al.,2008). However, the presence of oxygen in the bioreactor supports more planktonic biomass and therefore more flavins are produced. This makes the interpretation of electrochemical studies on only attached electrode biofilms difficult.

Also, oxygen regulates expression of a large set of genes in *S. oneidensis* and may interfere with controlled gene expression under anaerobic conditions. This indicates the need for improved strategies in the design of bioelectrochemical system that is efficient and also scalable to practical volumes. The main purpose of this study was to modify the bioreactor design to enable better sealing and minimize oxygen entrance into bioreactors. Increased control over anoxic conditions enables metabolic turnover to be compared to current generation providing accurate efficiency calculations.

The old bioreactor design consists of a teflon top sealed with silicone gasket and adhesive tape wrapped around teflon and glass cone. All the electrodes are attached to the teflon top using a teflon tape. Experiments were carried out to measure oxygen traces in the old bioreactors, and modifications were made to seal the bioreactor to make the design more efficient. PEEK (a thermoplastic polymer) was used to build tops for the modified design as it is more oxygen impermeable and robust to house stainless steel screws without any wear. PEEK provides longer life to the reactor and better control over the experimental conditions. Self-sealing gasket sheets are used to attach electrodes to the reactor top with the help of stainless steel screws. Eight stainless steel screws mounted on threaded ring helps in sealing the reactor.

Materials and Methods

Bacterial Culturing and growth conditions

Shewanella oneidensis strain MR-1 (JG274) was kindly provided by Dr. Jeffrey Gralnick. Cells were streaked from -80°C stock on LB agar plates, incubated at 30°C overnight. Single colonies were picked and grown in liquid aerobic LB medium at 30°C in a shaker at 250 rpm. Cells from aerobic culture were transferred (0.5% inoculum) to LB anaerobic medium. At mid exponential phase (~0.5- 0.6 OD), 1 ml of LB anaerobic culture was transferred to bioreactors containing 14 ml of SBM growth media. Shewanella basal media (SBM) used for growth contained the following (per liter): 0.225 g K_2HPO_4, 0.225 g KH_2PO_4, 0.46 g NaCl, 0.225 g $(NH_4)_2SO_4$, 0.117 g $MgSO_4.7H_2O$, 5 ml of vitamin mix (Hau et al., 2008), and 5 ml of trace mineral mix (Hau et al., 2008) supplemented with 0.05% casamino acids. 40 mM fumarate was used as electron acceptor under anaerobic conditions and biofilm growth stage in bioreactors, 60 mM D,L-lactate was used as the electron donor. Medium was buffered with 100 mM HEPES buffer, and adjusted to pH 7.2. Medium was flushed with argon gas for 30 min to remove oxygen in 100 mL bottles sealed with butyl rubber stoppers and aluminum crimps. SBM with 50 mM NaCl lacking any electron donors or acceptors was used as the reactor wash medium. SBM wash medium was supplemented with 30 mM lactate which served as the electron donor, 10 mM arabinose which served as the internal standard for HPLC analysis and 1 μM riboflavin for replenishing the washed flavins after media wash step. Luria-Bertani (LB) medium was used for aerobic cultivation and LB medium with added lactate (electron donor) and fumarate (electron acceptor) was used for anaerobic cultures.

Anaerobic LB medium was flushed with argon for 15 min to remove oxygen from the Balch tubes sealed with butyl rubber stoppers and aluminum crimps.

Reagents

Fumaric acid, DL-lactic acid, NaCl, arabinose, and riboflavin were obtained from Sigma-Aldrich.

Design of new bioreactor apparatus

Figure 2.1 depicts a schematic representation of modified three electrode bioreactor setup. The bioreactor consists of a graphite electrode measuring 0.5 cm x 3 cm x 1 mm attached to a platinum wire loop serving as the working electrode. A platinum wire attached to a glass tubing soldered with silver solder and copper wire for electrical connection serves as the counter electrode. Ag/AgCl reference electrodes were connected to bioreactors via a salt bridge assembled from glass tubing and a nanoporous Vycor frit (BioAnalytical Systems, West Lafayette, IN). Salt bridge was filled with 0.1 M KCl in 1% agarose and topped with 3M KCl. All the electrodes were fitted into a PEEK (a polyether ether ketone thermoplastic polymer) top placed onto 25 mL glass cone (Bioanalytical Systems, IN). The graphite working electrode was polished with 400 grit sandpaper, rinsed in 1 N HCl and cleaned by sonicating in deionized water twice for 10 min. The polished graphite electrode was then attached to a platinum wire loop using nylon bolt and nut (McMaster Carr). The bioreactors were monitored and potentials were maintained using a 16-channel VMP® potentiostat (Bio-Logic SA). Reactors were continuously flushed with humidified argon gas to maintain anoxic conditions, and a

temperature of 30°C was maintained by using a circulating water bath. Reactors were stirred continuously using stir bars.

Measuring the amount of Oxygen

A trace oxygen analyzer 3000 (Alpha Omega instruments) was used to measure the oxygen concentration in different settings of old bioreactor and new bioreactor. The inlet of the oxygen sensor was connected to the outlet of old bioreactor, humidifier apparatus and new bioreactor. The inlet of bioreactor and humidifier were connected to argon gas line.

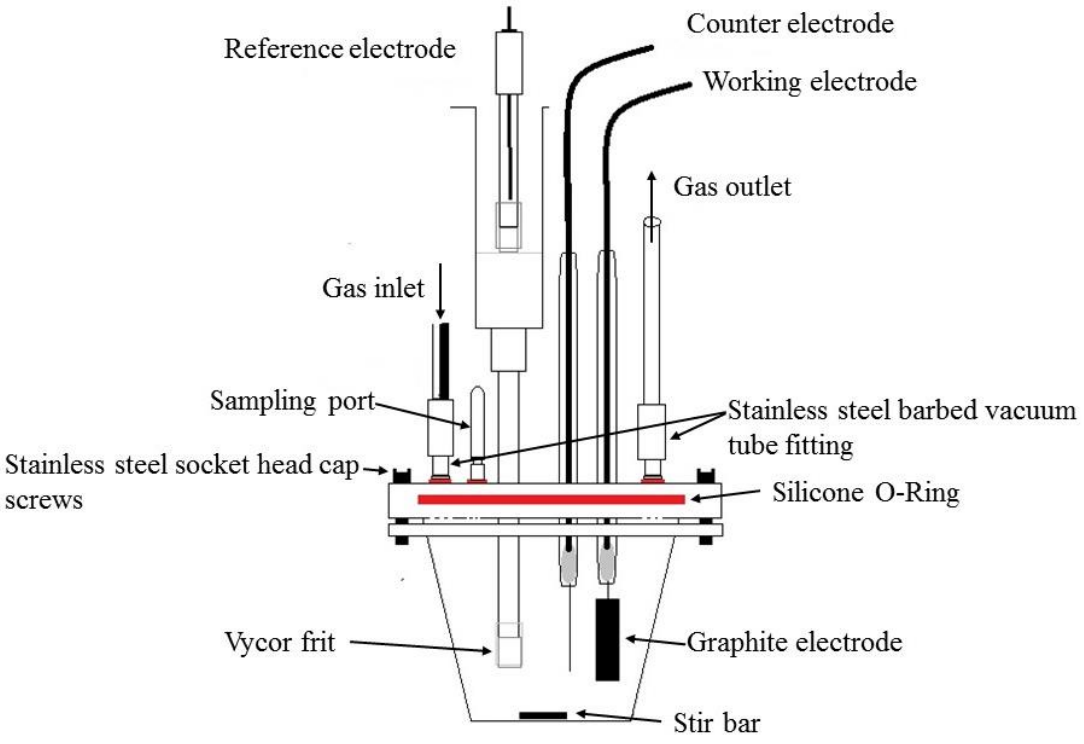


Figure 2.1 Modified 3-electrode bioreactor.

Electrochemical analysis

Electrochemical techniques were used to monitor the current production and biofilm formation of *S. oneidensis* MR-1 in modified bioreactors. Chronoamperometry (CA) is a technique that measures current production over time where the working electrode is poised at a set potential versus the reference electrode. The working electrode was poised at 0.240 V vs SHE (Standard Hydrogen Electrode). Bioreactors containing 14 mL SBM growth medium were inoculated with 1 mL of mid-exponential *S. oneidensis* culture (~0.5 – 0.6 OD) grown in LB anaerobic medium containing 20 mM lactate and 40 mM fumarate, and incubated for 100-110 hours until current production plateaued. Bioreactor media was then removed via sterile needles, washed with SBM lacking electron donor or acceptor. Bioreactor medium was replaced with SBM containing 30mM lactate, an internal standard (10 mM arabinose) and 1 μM riboflavin. Following medium replacement, current was monitored for 96 hours and HPLC samples were collected periodically and saved at -20°C. After 96 hours, cyclic voltammetry (CV) was conducted over a potential range from -0.555 V vs SHE to +0.450 V vs SHE at a scan rate of 1mV/sec.

HPLC analysis

Metabolites were quantified by high performance liquid chromatography (HPLC) on an Aminex 87H column using UV-Visible detector set at 210 nm and refractive index detector. The system (Shimadzu Scientific) consisted of an SCL-10A system controller, LC-10ATvp Liquid chromatograph, SIL-10AF autoinjector, CTO-10A column oven, RID-10A refractive index detector and SPD-10A UV-Vis detector. The separation of

compounds was performed with an Aminex HPx-87H guard column (Bio-Rad) and Aminex HPX-87H cation exchange column (Bio-Rad). The mobile phase was 0.015N H2SO4 with a flow rate of 0.4 ml/min. The column was maintained at 46°C and the injection volume was 50 µL.

Results and Discussion

The ability of *S. oneidensis* to utilize oxygen as a terminal electron acceptor led to a high planktonic growth in the previous bioreactors (Figure 2.3). Planktonic growth of *S. oneidensis* cells is possibly due to the presence of oxygen in the bioreactor headspace as well as dissolved oxygen in the medium.

Increasing the input flow rate of humidified argon gas could reduce oxygen levels in reactors (Figure 2.3). Increasing input gas flow rate also increase evaporation rate of media. Planktonic growth and medium evaporation impeded electrochemical analysis and efficiency calculations. Because of high biomass, more substrate was oxidized and more flavins accumulated leading to low efficiency. Theoretical calculations showed that 30-40% of electrons were consumed by oxygen respiration instead of going to the electrode assuming 10 $mL.min^{-1}$ gas flow rate and 10 ppm oxygen at a current of 50 µA (Figure 2.2), therefore decreasing the overall coulombic efficiency:

Coulombic efficiency (%) = moles of electrons transferred to electrode / moles of electrons produced by lactate oxidation to acetate.

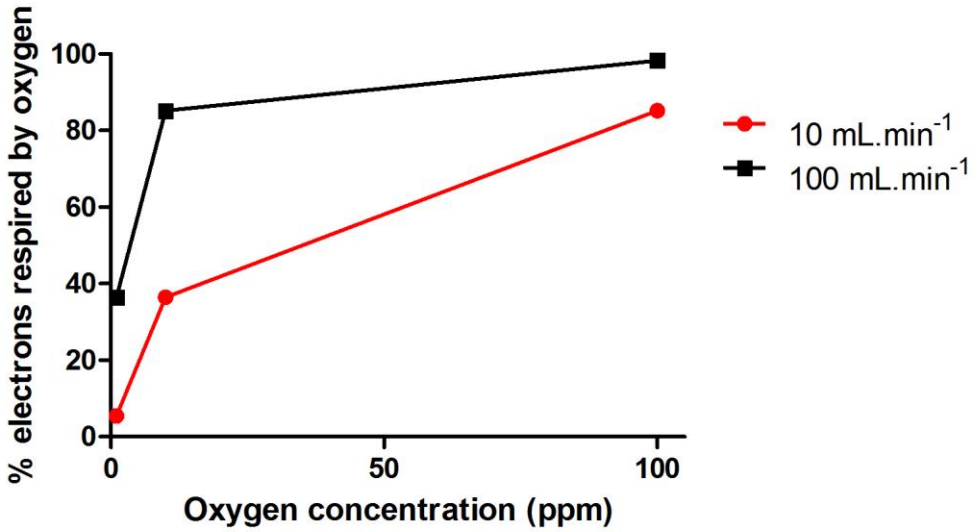


Figure 2.2 Theoretical calculation for oxygen respiration in old bioreactors. Current is assumed to be 50 µA.

To get an accurate value of oxygen concentration in the bioreactor headspace, the outlet of bioreactors was attached to a trace oxygen analyzer and at different reactor settings, headspace oxygen concentrations were measured. These oxygen measurements verified a need to better seal the reactors and redesign outlet and sampling ports.

The modified bioreactor design is made from PEEK instead of teflon in the old reactor setting. Old reactors used teflon tape to attach electrodes to the top, and had a gas inlet port and an outlet port/ sampling port. The modified reactors contain stainless steel barbed vacuum fittings (McMaster-Carr) threaded into PEEK tops, sealed with silicone gasket as gas inlet and outlet ports. The three electrodes are attached to PEEK top with the help of a silicone gasket cut in the form of a disc topped with a 1" PEEK disc tightened with the help of a stainless steel screw threaded at the center of the disc. A

separate sampling port (stainless steel threaded adapter) was housed on to the modified reactor tops. The modified reactor top and glass cone was sealed using a PEEK ring and stainless steel screws.

Different settings of bioreactors were tested for oxygen concentration in head space by changing the needles used in sample port (for old reactor setup), changing the flow rates of argon gas. First, the effect of flow rate on oxygen concentration was studied. Next, the effect of exhaust port on oxygen concentration was measured. Increasing the flow rate reduced the amount of oxygen measured in the headspace. (Figure 2.3). 37 ppm oxygen was measured to be present in the headspace of old bioreactor when a small needle was used in the sampling port with a high flow rate (1 $L.min^{-1}$) compared to >200 ppm oxygen in case of low flow rate. With big needles, 16 ppm oxygen was measured to be present at a high flow rate whereas >200 ppm oxygen was measured at low gas flow rates. Humidifier bottles that are fitted with two needles and a nylon membrane filter were also studied for oxygen measurements.

Oxygen measurements for new reactors were comparable to oxygen concentration directly from the gas lines. Oxygen in new reactor headspace when sampling port was sealed, outlet open to air with high as well as low flow rate of argon was measured to be 2.5 ppm (Figure 2.3). Oxygen concentration was not affected by the gas flow rate showing the effectiveness of new modified reactors. Lower gas flow rate measurements in new bioreactors also led to lower medium evaporation rates.

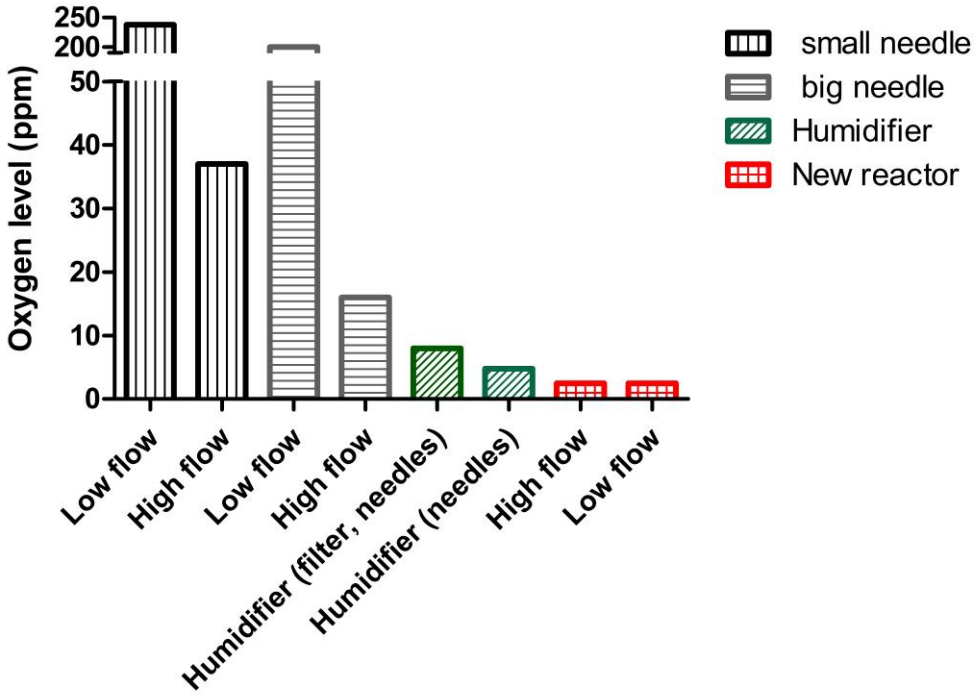


Figure 2.3 Comparison of oxygen concentration in different settings of old bioreactor versus new bioreactor. Different settings of bioreactors were tested for amount of oxygen in head space by changing the needles used in sample port, changing the flow rates of argon gas. In all cases, inlet to the reactor was directly connected to the gas line, inlet to the sensor was from one of the open glass rods fitted tightly on one of the electrode ports, and sampling port/ outlet of the old reactor was fitted with 18 Gauge or 22 Gauge needle and outlet for new reactor was open to air.

Modified bioreactors containing *S. oneidensis* showed no planktonic growth and resulted in higher current densities (Figure 2.5). When *S. oneidensis* was grown in old bioreactors, after approximately 110 hours of growth planktonic cells and pellicle formation is seen at the gas-liquid interface (Figure 2.4), whereas no planktonic growth is visible when *S. oneidensis* is grown in new modified bioreactor (Figure 2.4) after 110

hours of incubation and approximately same gas flow rates. High planktonic growth is one indicator of oxygen in the bioreactor headspace. Planktonic cell growth affects electrochemical analysis and gives misleading results.

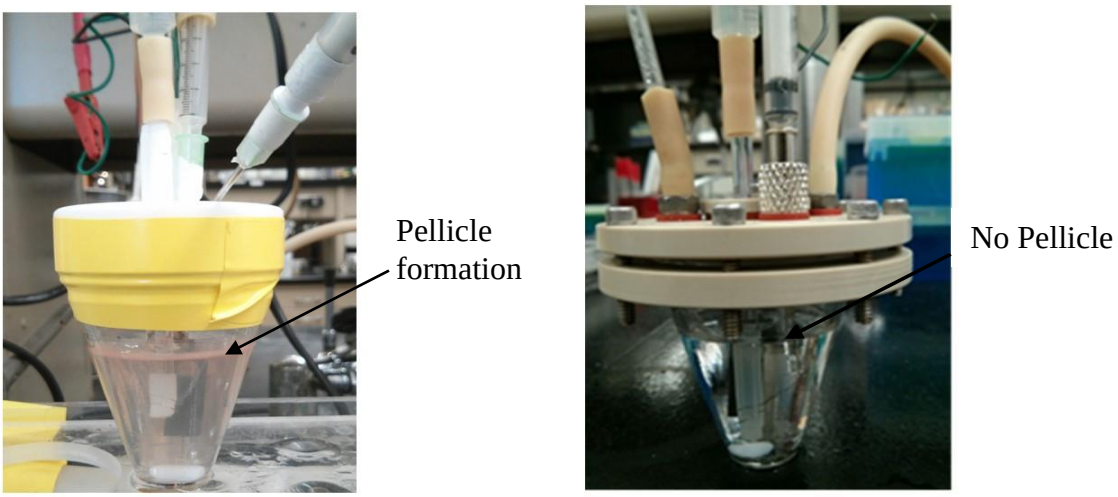


Figure 2.4 Planktonic cell growth in new versus old bioreactor seen after 110 hours of incubation. Pellicle is formed at the liquid and gas interface in old reactor showing presence of oxygen in the headspace where as no pellicle and planktonic growth is seen in new bioreactor.

Current densities were compared for *S. oneidensis* MR-1 cultures when grown in old vs new bioreactors. Maximum current density in old reactors was measured to be around 10 μA/cm^2 where as in new bioreactors, higher current density of 23-25 μA/cm^2 was achieved (Figure 2.5). Current (measured as μA) was measured over time indicative of attachment and growth of MR-1 on graphite electrodes.

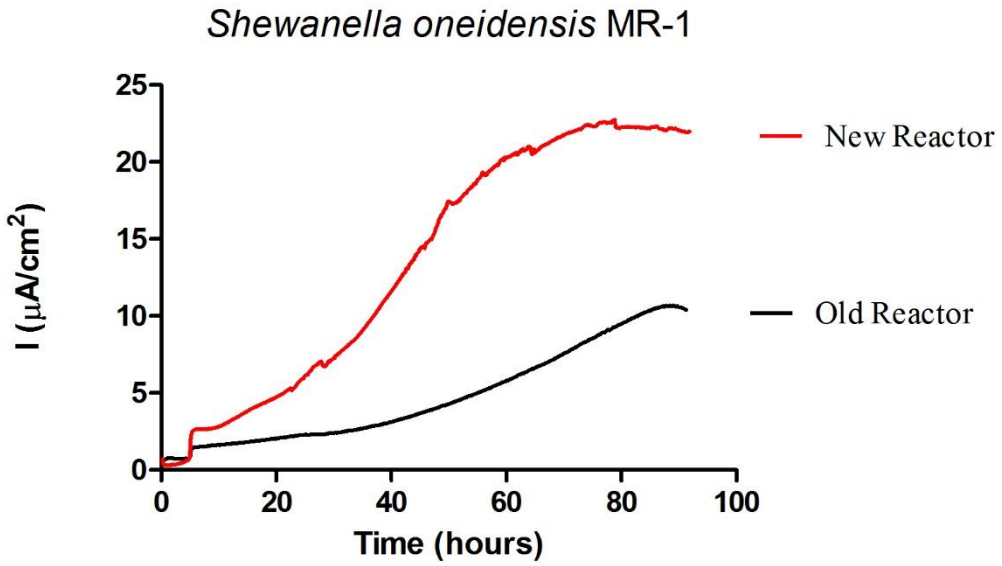


Figure 2.5 Comparison of electrode reduction rate and current densities in old reactor and new bioreactor.

To calculate the coulombic efficiency of *S. oneidensis* MR-1 in new bioreactors, bioreactors were set up in triplicates as previously described. Current production was measured over time and is represented as current density (μA/cm^2) on Y-axis and time (hr) on X-axis. Current was monitored until it plateaued around 110 hours, after which spent medium was removed and replaced with SBM containing 30mM lactate, 10 mM arabinose as internal standard for HPLC analysis and 1 μM riboflavin (Figure 2.6 A). Riboflavin was added after wash step because it is reported that current decreases by 70% in absence of riboflavin (Marsili et al., 2008). After replacing the bioreactor media, current production resumed to approximately same value suggesting no biofilm loss. Current was measured for another 100 hours and samples were collected for HPLC analysis periodically.

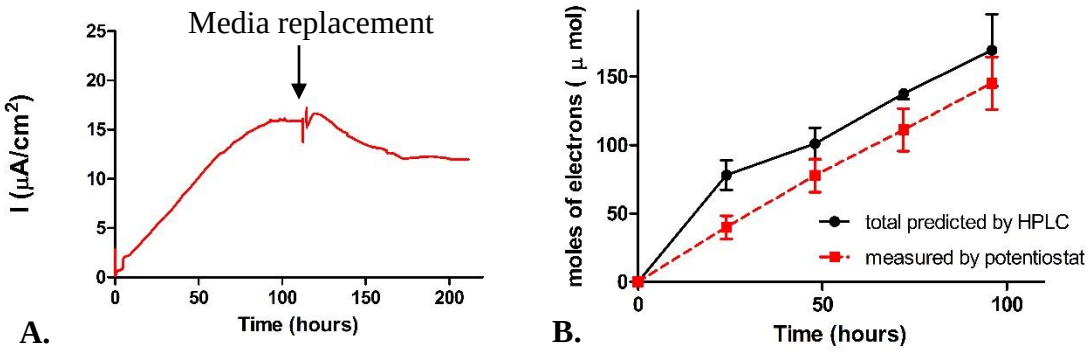


Figure 2.6 Growth of *S. oneidensis* MR-1 biofilm in new reactors. **A**: Current production was monitored for 110 hours for attachment and growth followed up by media replacement and current production was measured for 96 more hours. **B**: Metabolites were analyzed using HPLC and used for calculating total moles of electrons and measured electrons was recorded with potentiostat.

After collecting samples periodically, HPLC analysis was done and amount of lactate consumption and that of acetate production was measured. Fumarate, succinate, pyruvate and formate were not detected in the samples. The total charge passed into the electrodes by oxidation of lactate was calculated using stoichiometric metabolite balance (Figure 2.6 B). One mole of lactate is converted to one mole of acetate and four e^- equivalents. Total charge recorded by the potentiostat was converted to e^- equivalents and these values are reported in Figure 2.6 B

Total amount of acetate formed was used to calculate the total charge measured over time. This is the actual number of electrons released by the oxidation of lactate to acetate. Chronoamperometry (CA) analysis of *S. oneidensis* MR-1 was used to measure the amount of electrons taken up by the electrode over time (Figure 2.5 B). The following

equation was used to determine the coulombic efficiency of MR-1 biofilm in the new bioreactor set up.

Coulombic efficiency (%) = moles of electrons transferred to electrode / moles of electrons produced by lactate oxidation to acetate.

Coulombic efficiency was calculated to be 86.4 ± 10.37% for new reactors, higher than all the reported coulombic efficiency values: 9% by Biffinger et al (Biffinger et al, 2008), 56% by Lanthier et al (Lanthier et al, 2008). The new bioreactor provides a more controlled system to culture *S. oneidensis* mutants and can be used to isolate and enrich electrochemically active bacteria from other anaerobic subsurface environments as well as to study the process of extracellular electron transfer in other electrochemically active bacteria.

Chapter 3

Investigating the hydrogen metabolism in *Shewanella oneidensis* in bioreactors.

Introduction

Shewanella oneidensis MR-1 is a gram negative, facultative anaerobic γ-proteobacterium isolated from aquatic subsurface environments at oxic/anoxic conditions (Myers and Nealson, 1988). *S. oneidensis* can respire a diverse range of organic and inorganic electron acceptors including fumarate, dimethyl sulfoxide, trimethyl amine oxide, iron oxide, nitrate, manganese oxide, electrodes. The range of compounds utilized by *S. oneidensis* as electron donors is limited (Nealson and Scott, 2006). *S. oneidensis* can use hydrogen, and metabolic end products like lactate, pyruvate, formate and a few amino acids as electron donor (Gralnick and Newman, 2007). Hydrogen also serves as electron donor for reduction of insoluble metal oxides like iron, manganese, cobalt, chromium, and palladium. (Hau and Gralnick, 2007).

The ability to respire insoluble metals is linked to a series of multi-heme cytochromes across the membrane from cytoplasmic membrane to the extracellular side of the outer membrane. *S. oneidensis* utilizes Mtr pathway for respiration of insoluble metals and electrodes (Coursolle and Gralnick, 2010). Mtr pathway consisits of a tetra-heme cytochrome CymA, two deca-heme c-type cytochromes MtrA and MtrC, and a non-heme containing protein MtrB. Excess electrons from carbon source oxidation are transferred via the menaquinone pool to the cytoplasmic membrane-anchored tetrahaem *c*-type cytochrome CymA (Coursolle and Gralnick, 2010, Marritt et al, 2012).

Electrons are transferred from CymA to a periplasmic electron carrier protein, MtrA. MtrA facilitates the transfer of electrons to the extracellularly located MtrC via MtrB conduit embedded on the outer membrane (Coursolle and Gralnick, 2010). MtrC has been shown to be required for electrode reduction, flavin reduction, iron oxide and manganese reduction (Coursolle and Gralnick, 2010).

However, for reduction of certain metals like technetium (Tc) and palladium (Pd), hydrogen oxidation is also involved (De Windt, et al., 2005). Hydrogen oxidation takes place via hydrogenases. Hydrogenases catalyze the reversible reduction of protons into molecular hydrogen. Hydrogenases can be categorized into two types based on their active sites: [Ni-Fe] hydrogenases and [Fe-Fe] hydrogenases. [Ni-Fe] hydrogenases typically exist as heterodimers and are found in many facultative anaerobic bacteria whereas [Fe-Fe] hydrogenases may be present as monomer or heteromer and is found in strict anaerobic bacteria. [Ni-Fe] hydrogenases are involved in both uptake and release of hydroge but [Fe-Fe] hydrogenases are only involved in H_2 production (Spormann et al., 2007).

There are two putative hydrogenase clusters, *hydAB* (SO3920 to SO3926) and *hyaB* (SO2089 to SO2099) found in *S. oneidensis* genome (Hiedelberg et al., 2002). Structural features predicted HyaB to be a periplasmic [Ni-Fe] hydrogenase and HydA to be a periplasmic [Fe-Fe] hydrogenase. The gene arrangement of *hydAB* gene cluster consists of *hydA, hydB, hydG, hydE* and *hydF* genes with a putative formate dehydrogenase *(fdh)* gene situated in between *hydA* and *hydG* genes all expressed as a polycistronic unit (Spormann et al., 2007).

Spormann. et. al in 2007 found that these hydrogenases in *S. oneidensis* MR-1 are only expressed under anaerobic conditions. When *S. oneidensis* was grown with excess lactate or pyruvate as the electron donor, hydrogen formation was observed with the depletion of fumarate as electron acceptor when cell reached stationary phase. Addition of formate to the anaerobically growing *S. oneidensis* cells onset the hydrogen formation before depletion of fumarate as electron acceptor suggesting MR-1 can utilize hydrogen as well as produce hydrogen. However, the cell density with formate supplemented culture was found to be one-fourth of the cells growing without any formate addition.

In this study, we investigated the role of hydrogenases in current production in bioelectrochemical reactors. Hydrogenase double mutant was made by deleting two genes of the hydrogenases family in *S. oneidensis*: *hydA* (SO3920) and *hyaB* (SO2098). We hypothesized that *S. oneidensis* mutant lacking hydrogenases will make more current than wild type *S. oneidensis* MR-1 when grown on electrodes with lactate as the sole electron donor. The hypothesis is based on the assumption that there is no hydrogen formation in the *ΔhydA ΔhyaB* mutant, so the transfer of electrons can be solely directed to electrode respiration via the Mtr pathway under anoxic conditions in a bioreactor. Deleting hydrogenases blocks the transfer of electrons to hydrogenases diverting the flux of electrons to the electrode than being utilized for hydrogen formation as in case of wild type *S. oneidensis* resulting in an increase in the rate of electrode reduction.

Materials and Methods

Bacterial culturing and growth

Strains used in this study are listed below in Table 3.1. *Shewanella oneidensis* strain MR-1 (JG274) was kindly provided by Dr. Jeffrey Gralnick. Cells were streaked from -80°C stock on LB agar plates, incubated at 30°C overnight. Single colonies were picked and grown in liquid aerobic LB medium at 30°C in a shaker at 250 rpm. Cells from aerobic culture were transferred (0.5% inoculum) to LB anaerobic medium. At mid exponential phase (~0.5- 0.6 OD), 1 ml of LB anaerobic culture was transferred to bioreactors containing 14 ml of SBM growth media. Shewanella basal media (SBM) used for growth contained the following (per liter): 0.225 g K_2HPO_4, 0.225 g KH_2PO_4, 0.46 g NaCl, 0.225 g $(NH_4)_2SO_4$, 0.117 g $MgSO_4.7H_2O$, 5 ml of vitamin mix (Hau et al., 2008), and 5 ml of trace mineral mix (Hau et al., 2008) supplemented with 0.05% casamino acids. 40 mM fumarate was used as electron acceptor under anaerobic conditions and biofilm growth stage in bioreactors, 60 mM D,L-lactate was used as the electron donor. Medium was buffered with 100 mM HEPES buffer, and adjusted to pH 7.2. Medium was flushed with argon gas for 30 min to remove oxygen in 100 mL bottles sealed with butyl rubber stoppers and aluminum crimps. SBM with 50 mM NaCl lacking any electron donors or acceptors was used as the reactor wash medium. SBM wash medium was supplemented with 30 mM lactate which served as the electron donor, 10 mM arabinose which served as the internal standard for HPLC analysis and 1 µM riboflavin for replenishing the washed flavins after media wash step. Luria-Bertani (LB) medium was used for aerobic cultivation and LB medium with added lactate (electron donor) and

fumarate (electron acceptor) was used for anaerobic cultures. Anaerobic LB medium was flushed with argon for 15 min to remove oxygen from the Balch tubes sealed with butyl rubber stoppers and aluminum crimps.

Strain or Plasmid	**Characteristics**	**Reference/Source**
S. oneidensis strain MR-1	Isolated from L. Oneida, NY	Venkateswaran, Moser et al (1999)
JG2642	*S. oneidensis* MR-1, ΔhydA, ΔhyaB	This study
WM3064	DAP auxotroph *E. coli* conjugal donor strain	Saltikov and Newman (2003)
Plasmid		
pSMV3	9.1 kb suicide vector; *oriR6K*, mobRP4, sacB, Km^R	Saltikov and Newman (2003)

Table 3.1 Strains and plasmids used in this study.

Reagents

Fumaric acid, D L-lactic acid, NaCl, arabinose, riboflavin, potassium phosphate dibasic, potassium phosphaste monobasic, ammonium sulfate, and magnesium sulfate were obtained from Sigma-Aldrich. Restriction enzymes, T4 DNA ligase were obtained from Agilent Technologies. For PCR cleanup, gel extraction and plasmid preparation, QIAquick PCR Purification Kit, QIAquick Gel Extraction Kit and QIAprep Spin Miniprep Kit from Qiagen were used respectively.

Construction of deletion Strains

Oligonucleotide primers used to amplify regions of the MR-1 chromosome for deletion constructs are listed in Table 3.2. Regions of approximately 500 base pairs both up and downstream of the target deletion region were amplified using primers hyaB-UF/hyaB-UR, hyaB-DF/hyaB-DR, hydA-UF/hydA-UR, and hydA-DF/hydA-DR with high fidelity polymerase Pfu Ultra (Agilent Technologies, Santa Clara, CA). Following restriction digest, up and downstream fragments were gel purified and cloned into the suicide vector pSMV3. Deletion constructs were introduced into MR-1 via conjugal transfer using *E. coli* donor strain WM3064 (Saltikov and Newman, 2003). In-frame gene deletions for *hydA* and *hyaB* were generated by homologous recombination as described previously (Saltikov and Newman, 2003). Plasmid constructs and deletion strains were sequence verified using primers hyaB-SP1/hyaB-SP-2 and hydA-SP1/hydA-SP2 at the University of Minnesota Genomics Center.

Primer name	**Sequence**
Primers for *hydA* deletion:	
hydA-UF	GCATGGGCCCGCATTATCAATTCACCATAAACCC
hydA-UR	GCATACTAGTATTAATCTTGATCAGCCC
hydA-DF	GCATACTAGTGTGAAATCAGCCTCTGTC
hydA-DR	GCATGAGCTCTTTTGCTAGGCTGTCGTCCTTG
Primers for *hyaB* deletion:	

hyaB-UF	GCATGGGCCCGTGGCCGTTTTGATGCAG
hyaB-UR	GCATACTAGTTTTCAGTATGACTTCAATAAC
hyaB-DF	GCATACTAGTGATGCTGTCAATGCCCTG
hyaB-DR	GCATGAGCTCATGCGGGTTTCAGAATGG

Primers for sequence verification:

hydA-SP1	TTCGACTCTACCTATGAAGCAATTAC
hydA-SP2	GATGTGCACATCATAGGTTAGCTG
hyaB-SP1	GTCAGCAAACCCGTGATTAACTTAG
hyaB-SP2	GATCCAACCTTGTACTAATACATCCGT

Table 3.2 Primers used for amplifying *hydA* and *hyaB* genes for construction deletion mutants and primers for sequence verification of deletion strains.

Bioreactor set-up

The bioreactor consists of a graphite electrode measuring 0.5 cm x 3 cm x 1 mm attached to a platinum wire loop serving as the working electrode. A platinum wire attached to a glass tubing soldered with silver solder and copper wire for electrical connection serves as the counter electrode. Ag/AgCl reference electrodes were connected to bioreactors via a salt bridge assembled from glass tubing and a nanoporous Vycor frit (BioAnalytical Systems, West Lafayette, IN). Salt bridge was filled with 0.1 M KCl in 1% agarose and topped with 3M KCl. All the electrodes were fitted into a PEEK (a polyether ether ketone thermoplastic polymer) top placed onto 25 mL glass cone (Bioanalytical Systems, IN). The graphite working electrode was polished with 400 grit

sandpaper, rinsed in 1 N HCl and cleaned by sonicating in deionized water twice for 10 min. The polished graphite electrode was then attached to a platinum wire loop using nylon bolt and nut (McMaster Carr). The bioreactors were monitored and potentials were maintained using a 16-channel VMP® potentiostat (Bio-Logic SA). Reactors were continuously flushed with humidified argon gas to maintain anoxic conditions, and a temperature of 30°C was maintained by using a circulating water bath. Reactors were stirred continuously using stir bars. Outlet port of the bioreactor was sealed by fitting a sealed syringe. Glass tubing encasing the cathode was used as outlet port.

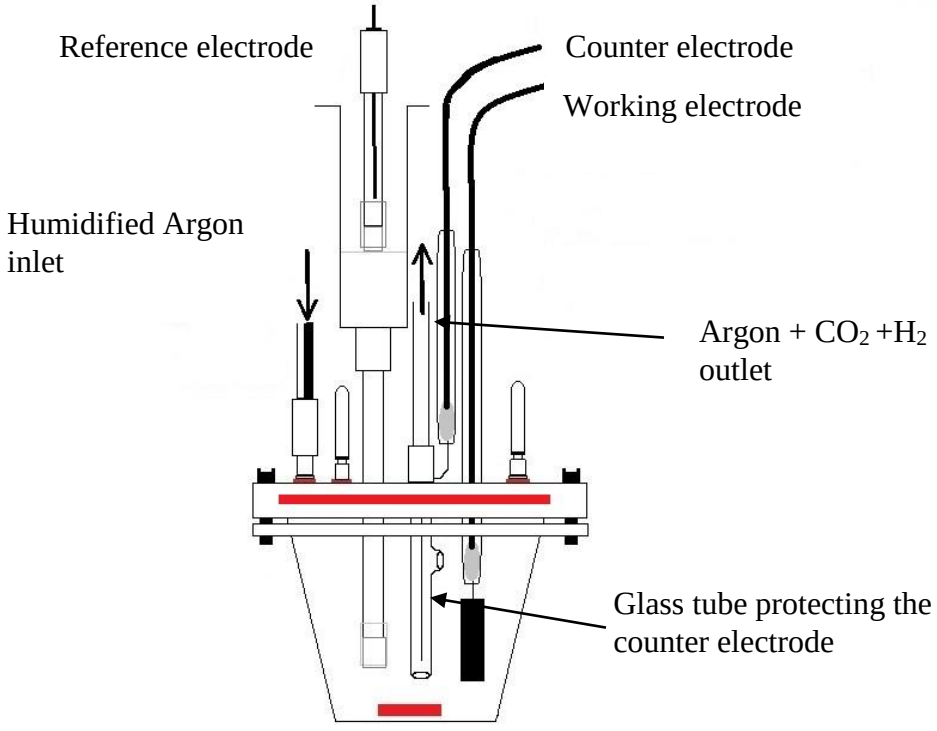


Figure 3.1 Anaerobic single chamber 3-electrode bioreactor with a glass tubing protecting the hydrogen produced at cathode from mixing into the media.

Electrochemical analysis

Electrochemical techniques were used to monitor the current production and biofilm formation of *S. oneidensis* MR-1 in modified bioreactors. Chronoamperometry (CA) is a technique that measures current production over time where the working electrode is poised at a set potential versus the reference electrode. The working electrode was poised at 0.240 V vs SHE (Standard Hydrogen Electrode). Bioreactors containing 14 mL SBM growth medium were inoculated with 1 mL of mid-exponential *S. oneidensis* culture (~0.5 – 0.6 OD) grown in LB anaerobic medium containing 20 mM lactate and 40 mM fumarate, and incubated for 100-110 hours until current production plateaued. Bioreactor media was then removed via sterile needles, washed with SBM lacking electron donor or acceptor. Bioreactor medium was replaced with SBM containing 30mM lactate, an internal standard (10 mM arabinose) and 1 µM riboflavin. Following medium replacement, current was monitored for 96 hours and HPLC samples were collected periodically and saved at -20°C. After 96 hours, cyclic voltammetry (CV) was conducted over a potential range from -0.555 V vs SHE to +0.450 V vs SHE at a scan rate of 1mV/sec. The electrodes were harvested for BCA protein assay.

Fe(III) citrate reduction Assay

Real-time Fe(III) citrate reduction experiments were carried out in 96-well plates as previously described with the following modifications (Chan et al, 2015). Briefly, strains cultured for 12 hours in anoxic SBM containing 20 mM lactate and 50 mM fumarate were washed and re-suspended in SBM to a final optical density measured at

600 nm (OD_{600}) of ~0.1. Cells were added to 96-well plates followed by a 10:1 volume of buffered solution containing sodium DL-lactate (10 mM), Fe(III) citrate (5 mM), NaCl (50 mM), K_2HPO_4 (1.3 mM), KH_2PO_4 (1.7 mM), $(NH_4)SO_4$ (1.7 mM), $MgSO_4$ (0.5 mM), HEPES (100 mM), and FerroZine reagent (2g/L). All preparations and incubations were carried out in an OMNI-LAB glove box (Vacuum Atmospheres Company) under a nitrogen atmosphere. Fe(II) concentration was assayed at 1 minute intervals at 625 nm using a SpectraMax M2 multimode microplate reader (Molecular Devices) over four hours. Fe(II) concentration was calculated using a standard curve generated with ferrous sulfate dissolved in 0.5 N hydrochloric acid.

HPLC analysis

Metabolites were quantified by high performance liquid chromatography (HPLC) on an Aminex 87H column using UV-Visible detector set at 210 nm and refractive index detector. The system (Shimadzu Scientific) consisted of an SCL-10A system controller, LC-10ATvp Liquid chromatograph, SIL-10AF autoinjector, CTO-10A column oven, RID-10A refractive index detector and SPD-10A UV-Vis detector. The separation of compounds was performed with an Aminex HPx-87H guard column (Bio-Rad) and Aminex HPX-87H cation exchange column (Bio-Rad). The mobile phase was 0.015N H2SO4 with a flow rate of 0.4 ml/min. The column was maintained at 46°C and the injection volume was 50 µL.

Results and Discussion

When wild type *S. oneidensis* MR-1 cells were grown anaerobically in single chambered bioreactors with lactate as the electron donor with a shielded and a non-shielded cathode, we observed different growth rates. Wild type *S. oneidensis* MR-1 growing anaerobically in bioreactors with a shielded cathode produced a significantly less current density compared to wild type MR-1 growing in bioreactors without any shielded cathode (Figure 3.2). The difference in current densities suggests the possibility of hydrogen recycling between cathode and MR-1 cells. *S. oneidensis* has the ability to utilize hydrogen as the electron donor as well as respire hydrogen as an electron acceptor (Figure 3.2). Shielded cathodes allowed hydrogen produced at the cathode to escape to the headspace due to its low solubility (Figure 3.3) (Lee et al, 2009)

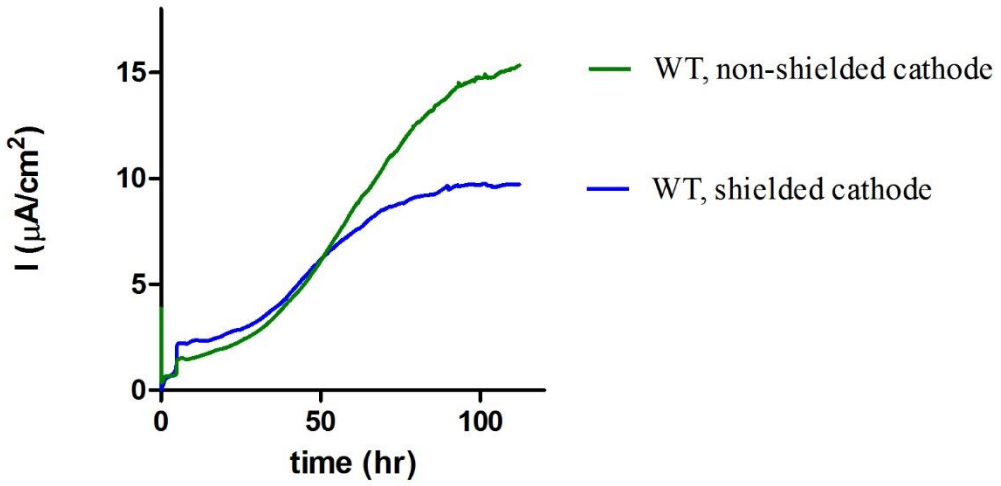


Figure 3.2 Chronoamperometry and growth of wild type *S. oneidensis* MR-1 in bioreactors on electrodes. The graph shows difference in final current density of same strain under two different conditions. Higher current density is observed when S. oneidensis is grown on electrodes poised at + 0.240 mV in a single chamber bioelectrochemical system under anaerobic conditions as compared to the condition when the cathode is shielded to prevent hydrogen recycling in the single chambered bioreactor.

To test whether hydrogen recycling is the factor causing change in current density and growth rate on electrodes, we constructed markerless-in-frame double deletion mutant of the two *S. oneidensis* hydrogenase genes, *hydA* (SO3920) and *hyaB* (SO2098).

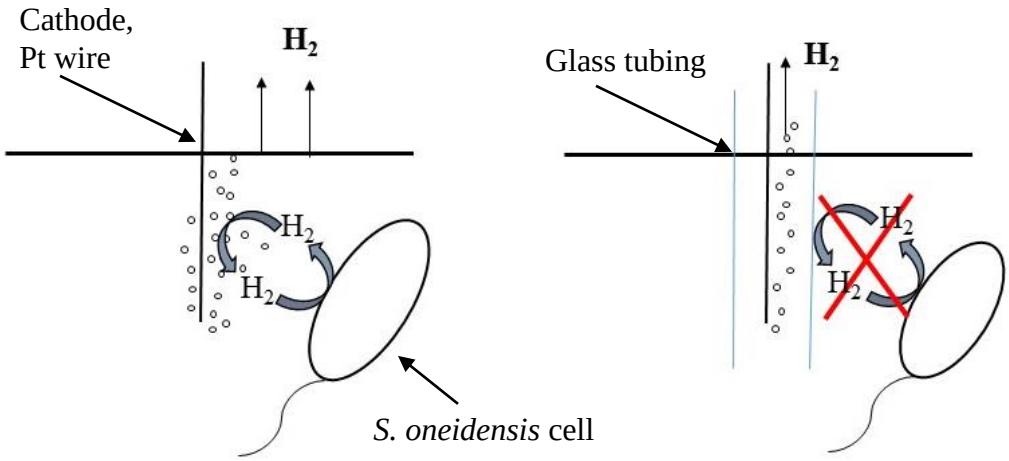
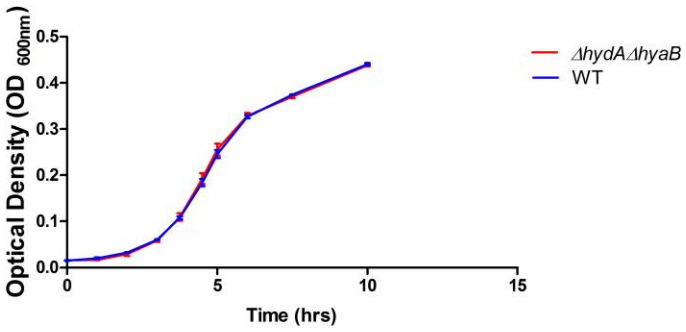


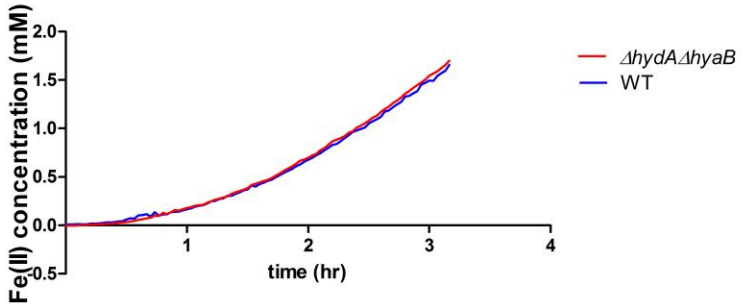
Figure 3.3 Schematic diagram of hydrogen recycling between *S. oneidensis* and cathode in a bioelectrochemical system. When the cathode is shielded with a glass tubing, cells cannot utilize hydrogen for growth and electrode reduction.

WT and hydrogenase mutant strains were grown anaerobically in SBM supplemented with lactate as electron donor and fumarate as electron acceptor. WT and hydrogenase mutant strains showed same growth rate under fumarate respiration conditions (Figure 3.4.A) and ferric citrate reduction conditions (rate 13.27±5.63 mM Fe(II)/hr/OD for 274, 13.81±3.27 mM Fe(II)/hr/OD for Δ*hyaB*Δ*hydA* , respectively) (Figure 3.4.B). These results suggest no phenotypic difference between WT and hydrogenase mutant under fumarate and ferric citrate reduction conditions.

A.



B.



C.

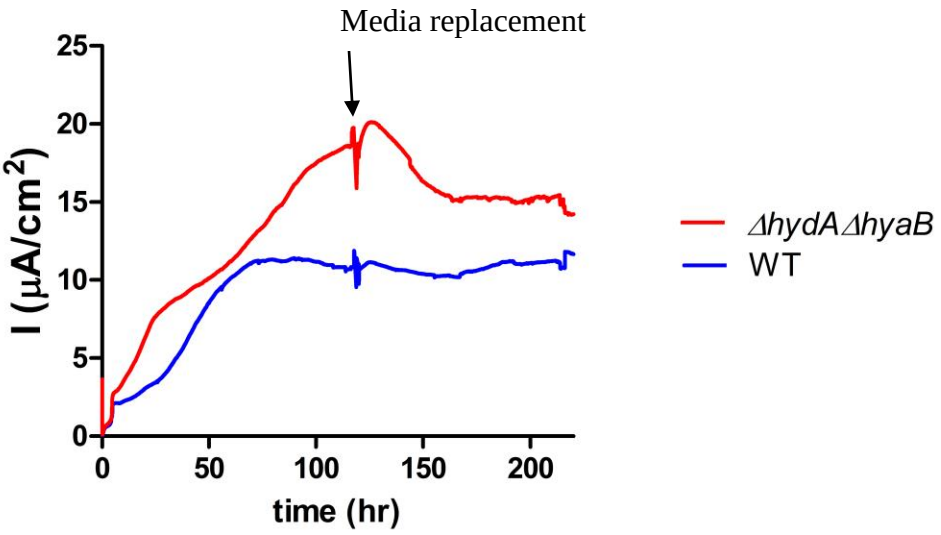


Figure 3.4 Mutant phenotype under different respiratory conditions. A: Growth measured by an increase in optical density over time. B: Real time ferric citrate reduction

rate measured by an increase in the rate of accumulation of Fe(II) over time determined by Ferrozine assay. C: Rate of poised electrode reduction shown as the rate of increase in current production over time in bioreactors with shielded cathode.

The difference in current densities between WT and hydrogenase mutant cells when grown anaerobically in a single chambered three electrode bioelectrochemical system with shielded cathodes suggested that hydrogen metabolism is also involved in current production. After current plateaued, bioreactor media was removed and washed with SBM media containing no electron donor or acceptor, and media was again replaced containing 30 mM lactate as the sole electron donor, 1 μM riboflavin, 10 mM arabinose as an internal standard for HPLC measurements.

The hydrogenase mutants produced more current density (~18 $\mu A/cm^2$) over time compared to MR-1 (~11 $\mu A/cm^2$) when grown in bioreactors. Mutant cells do not possess the ability to produce hydrogen and respire hydrogen as both the hydrogenases genes have been deleted. In contrast, WT *S. oneidensis* cells when grown anaerobically in bioreactors with shielded cathode can produce hydrogen which diffuses out of the reactor preventing cells from utilizing hydrogen as electron donor as the cathode is shielded. We observed that WT cells make less current over time compared to hydrogenase mutant indicating that the electron flow out of the cell also depends on the hydrogen metabolism.

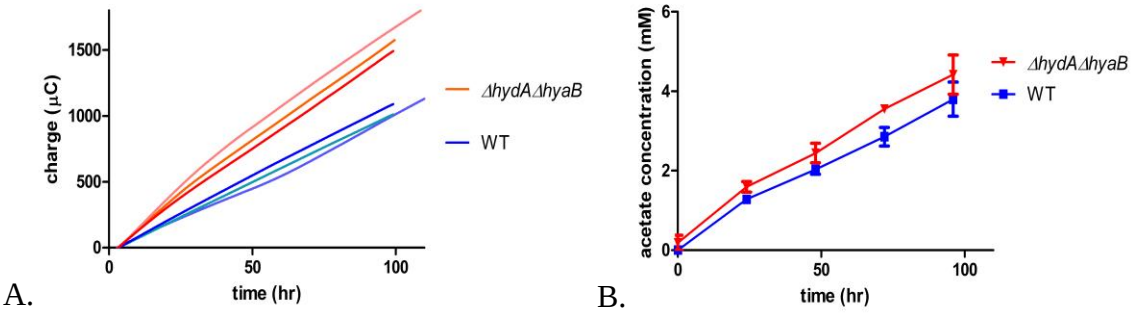


Figure 3.5 Charge measured over time A. Charge recorded by the potentiostat. *S. oneidensis ΔhydA/ΔhyaB* mutants (in red) show higher charge values recorded over time as compared to WT (in blue). Different lines shows the different replicates. B. HPLC analysis results of acetate production in WT and hydrogenase mutant reactors. There is no significant difference in the rate of acetate production in both reactors showing that both the strains utilize lactate at approximately same rates.

Bioreactors with *ΔhydA ΔhyaB* (JG2642) mutant recorded more charge over time when lactate was used as the sole electron donor but bioreactors with WT *S. oneidensis* recorded less charge over time (Figure 3.5). The different charge values suggests the possibility: mutant cells are oxidizing lactate faster than WT cells. To test this hypothesis, HPLC analysis of metabolites was used to determine the rates of lactate oxidation to acetate. The rate of acetate production in both the reactors were observed to be similar (figure 3.5 B) suggesting the difference in current production is not due to different lactate oxidation/ acetate production rate.

There was a significant difference in the coulombic efficiency of bioreactors with hydrogenase mutants as compared to bioreactors with wild type *S. oneidensis*. The

following equation was used to determine the coulombic efficiency of MR-1 biofilm in the new bioreactor set up.

Coulombic efficiency (%) = moles of electrons transferred to electrode / moles of electrons produced by lactate oxidation to acetate.

Figure 3.6 shows coulombic efficiency of bioreactors with *ΔhydA ΔhyaB* mutant and WT. The coulombic efficiency of WT cells in bioreactors with shielded cathodes was measured only about 51.93 ± 10.35 %. The efficiency increased when hydrogenase genes were deleted and mutants were studied in bioreactors with shielded cathode.

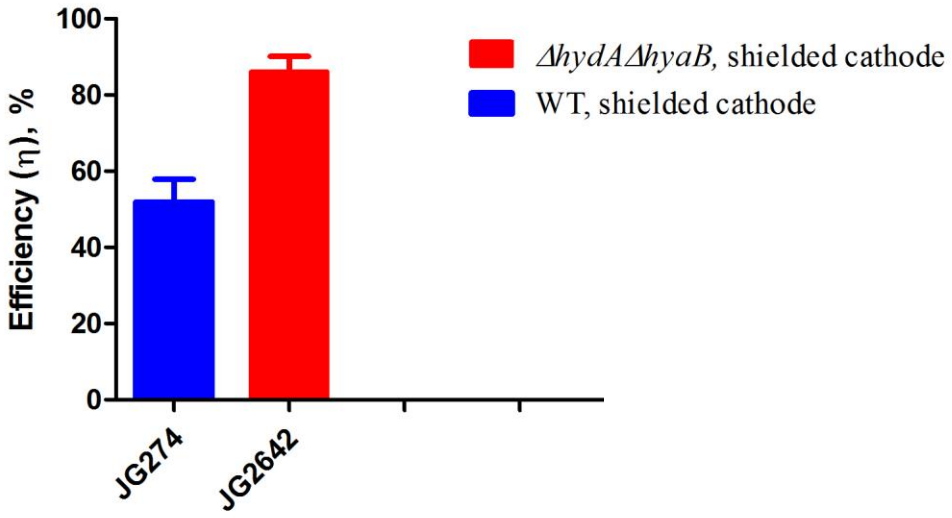


Figure 3.6 Coulombic efficiency measured for *S. oneidensis* bioreactors with WT (shielded cathode) and *ΔhydA ΔhyaB* mutants (shielded cathode).

In summary, this study showed that hydrogen metabolism also regulates the extracellular electron transfer pathway in *S. oneidensis* when poised electrodes are used as the sole electron acceptor. This study shows evidence for hydrogen cycling in *S. oneidensis* reactors as hydrogen can both be a donor or acceptor, which reduces the overall coulombic efficiency. *ΔhydA/ΔhyaB* mutants shows hydrogen production is a key source of electron loss in MR-1 bioreactors. Deleting hydrogenases from the *S. oneidensis* provides one of the methods for improving the overall efficiency of microbial fuel cells by funneling the electron flux towards anode.

Chapter 4

Determining the evolution route of pGUT2 and pGUT2PET plasmid by sequencing and repairing the plasmid

Introduction

Redox balance and thermodynamics are very important to all chemical and biochemical reactions. All reactions on this planet must be redox balanced. Alternative routes are often used by the microorganisms to achieve redox balance forming side products. Purification of the desired compounds becomes necessary and it adds to the final cost of the product, whereas if an obligatory production pathway is used, high yields of product can be achieved at lower costs.

This concept of subjecting microorganism to an obligatory production pathway was used to engineer *S. oneidensis* for the production of ethanol (Flynn et al., 2010). Anaerobic respiratory pathways in *S. oneidensis* enabled the coupling of oxidative metabolism with electrode respiration to balance redox reactions. The extracellular electron transfer ability of *Shewanella* balances the redox reactions by transferring excess electrons out of the cells to electrodes. An anaerobic respiratory pathway was introduced in *S. oneidensis* that utilized desired metabolic pathways only, limiting growth-associated cell mass. Poised electrode serving as an electron acceptor eliminates the need to provide soluble electron acceptors in the medium and enables cells to make only the desired product and no other side products (Flynn et al., 2010).

Glycerol was chosen as the carbon source and electron donor. It is the byproduct of biodiesel production and has limited uses. Using engineered pathways, glycerol can be used for conversion into more value added chemicals like succinate, ethanol etc. The engineered pathway for metabolic oxidation of glycerol to ethanol in *S. oneidensis* is shown in Figure 4.1

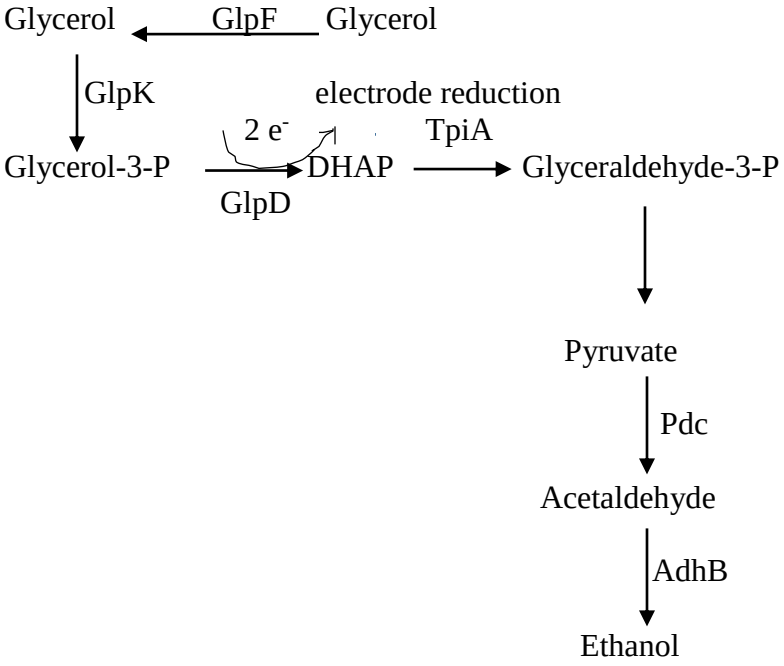


Figure 4.1 Pathway for ethanol production from glycerol in MR-1. GlpF stands for glycerol facilitator, GlpK: glycerol kinase, GlpD: glycerol-3-phosphate dehydrogenase, TpiA: triose phosphate isomerase, Pdc: pyruvate decarboxylase, and AdhB: alcohol dehydrogenase B. Two excess electrons generated in the conversion of glycerol-3-phosphate to dihydroxyacetone phosphate (DHAP) are transferred to the electrode to generate current.

Two genetic modules were engineered into *S. oneidensis* to allow for glycerol utilization and ethanol production in a manner that would feed directly into this electrode respiration machinery (Flynn et al, 2010). The glycerol utilization module from *E. coli*

and the ethanol production module from *Z. mobilis* were combined with native metabolic pathways of *S. oneidensis* for non-redox balanced conversion of glycerol to ethanol. Four genes from *E. coli*: *glpF*, *glpK*, *glpD*, and *tpiA* which encode a glycerol facilitator, a glycerol kinase, and a membrane-bound quinone-linked glycerol-3-phosphate dehydrogenase, and triose phosphate isomerase respectively, enabled *S. oneidensis* with the ability to utilize glycerol as sole carbon and energy source. The glycerol utilization module was cloned into a pBBR1 MCS-2 plasmid, named as pGUT2. Previous studies showed that this plasmid was acclimatized aerobically to grow faster is glycerol by 10 transfers of S. oneidensis containing the pGUT2 plasmid on aerobic SBM containing 40mM glycerol as the sole carbon and energy source. (Flynn et al., 2010). Adaptation experiments were also conducted under anaerobic conditions with glycerol as the electron donor and fumarate as electron acceptor. After ten transfers, the plasmids were isolated and transformed into WT *S. oneidensis*. Results suggested that mutations occurred on the plasmid enabling faster on glycerol. Glycerol facilitator (GlpF) is an inner membrane protein which facilitates the transfer of glycerol from outside of the cell to inside (Stroud et al, 2003). GlpF is a member of aquaporin protein superfamily which have highly conserved loops in cytoplasmic region and an extracellular loop. It interacts with glycerol kinase (GlpK) to stimulate glycerol phosphorylation which prevents the backflow of glycerol from the cell (Voegele et al., 1992, Henin et al., 2008). GlpD then converts glycerol 3-phosphate to dihydroxyacetone phosphate, generating electrons which are funneled via CymA to electrodes in *S. oneidensis*. TpiA enzyme isomerizes dihydroxyacetone phosphate to glyceraldehyde 3-phophate, which enters native

metabolic pathways in *S. oneidensis*. An ethanol module containing genes: pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) from *Z. mobilis* were first cloned into pET plasmid and then cloned to pGUT2 plasmid, making pGUT2PET plasmid.

The following studies aim to determine the evolution route of pGUT2 and pGUT2PET plasmids for faster growth in glycerol in *S. oneidensis* so that site directed mutagenesis strategies could be applied for improving the turnover rates of the enzymes involved in the study. The study also aim to repair the mutations in glycerol facilitator gene that suggests all the adaptive mutations to occur in glycerol kinase gene in order to compensate for the loss of function of the glycerol facilitator.

Materials and Methods

Bacterial culturing and growth conditions

Strains used in this study are listed below in Table 4.1. All *Shewanella* incubations were done at 30°C and *E.coli* incubations were done at 37°C and shaken continuously at 300 rpm. Overnight aerobic cultures were inoculated from single colonies freshly streaked from frozen culture stocks into LB media supplemented with 50 $\mu g \cdot mL^{-1}$ of Kanamycin. Cells from aerobic culture were transferred (1% inoculum) to LB anaerobic medium. At mid exponential phase (~0.5- 0.6 OD), 1 ml of LB anaerobic culture was transferred to bioreactors containing 14 ml of SBM growth media. *Shewanella* basal media (SBM) was used for growth and contained the following (per liter): 0.225 g K_2HPO_4, 0.225 g KH_2PO_4, 0.46 g NaCl, 0.225 g $(NH_4)_2SO_4$, 0.117 g

$MgSO_4.7H_2O$, 5 ml of vitamin mix (Hau et al., 2008), and 5 ml of trace mineral mix (Hau et al., 2008), supplemented with 0.05% casamino acids. 40 mM fumarate was used as electron acceptor under anaerobic conditions and biofilm growth stage, 40 mM glycerol or 60 mM D,L-lactate was used as the electron donor. Medium was buffered with 100 mM HEPES, and adjusted to pH 7.2, medium was flushed with argon gas for 30 min to remove oxygen in bottles sealed with butyl rubber stoppers and aluminum crimps. The above stated medium with 50 mM NaCl and no added other electron acceptor and electron donor was used as the reactor wash medium. SBM wash medium was supplemented with 40 mM glycerol which served as the electron donor, 10 mM arabinose which served as the internal standard for HPLC analysis and 1 µM riboflavin to replenish flavins after the media wash. Luria-Bertani (LB) medium was used for aerobic cultivation and LB medium with added glycerol (electron donor) and fumarate (electron acceptor) was used for anaerobic cultures. Anaerobic LB medium was flushed with argon for 15 min to remove oxygen from the Balch tubes sealed with butyl rubber stoppers and aluminum crimps.

Strain or Plasmid	**Characteristics**	**Reference/Source**
S. oneidensis strain MR-1	Isolated from L. Oneida, NY	Venkateswaran, Moser et al. (1999)
JG1083	*S. oneidensis* MR-1, pGUT2PET, plasmid evolved aerobically.	Flynn et al. (2010)
JG1327	*E. coli* UQ950, pGUT2PET	Gralnick lab culture collection

JG993	*E. coli* UQ950, pGUT2	Gralnick lab culture collection
JG2069	*E. coli* UQ950, pGUT2_A	Gralnick lab culture collection
G. subterraneus Red1	Isolated from production water of Redwash oilfield, UT. Stock culture was found contaminated and reisolated.	Greene et al. (2009), Badalamenti et al. (2015)
E. coli K12	Wild type	Gralnick lab culture collection
E. coli UQ950	E. coli DH5α host for cloning	Saltikov and Newman (2003)
E. coli WM3064	DAP auxotroph *E. coli* conjugal donor strain	Saltikov and Newman (2003)
Plasmids		
pGUT2	pBBR1MCS-2 containing *glpD, glpF, glpK, tpiA* (cloned from *E. coli* K12)	Flynn et al. (2010)
pGUT2_A	pBBR1MCS-2 containing *glpD, glpF, glpK, tpiA* (cloned from *E. coli* K12), adapted version.	Flynn et al. (2010)
pGUT2PET	pBBR1MCS-2 containing *glpD,*	Flynn et al. (2010)

	glpF, glpK, tpiA (cloned from *E. coli* K12), *pdc* and *adh* (cloned from pLOI297, ATCC 11303)	
pGUT2PET_O	pGUT2PET plasmid evolved aerobically in *S. oneidensis* MR-1	Flynn et al. (2010)
pGUT2PET_A	pGUT2PET plasmid evolved anaerobically from pGUT2PET_O in *S. oneidensis* MR-1.	This study
pGUT2_Geo	pBBR1MCS-2 containing *glpD, tpiA* (cloned from *E.coli* K12), *glpF* and *glpK* (cloned from *Glk. subterraneus*)	This study

Table 4.1 Strains and plasmids used in this study.

Reagents

All restriction enzymes and Alkaline Phosphatase were obtained from New England Biolabs. Phusion HSII polymerase and T4 ligase were obtained from Epicentre (Madison, WI). For PCR cleanup and gel extraction, PureLink PCR purification kit, PureLink gel extraction kit from Invitrogen were used. For plasmid preparation, Wizard spin miniprep kit from Promega was used. Glycerol, fumaric acid, D L-lactic acid and riboflavin were obtained from Sigma-Aldrich.

Anaerobic acclimatization of pGUT2PET on glycerol

Wild type *S. oneidensis* MR1 strain with pGUT2PET_O plasmid (JG1083) was used for evolution of pGUT2PET plasmid anaerobically to select for faster growth on glycerol. JG1083 was grown overnight aerobically in LB supplemented with 50 $\mu g \cdot mL^{-1}$ kanamycin from single colonies freshly streaked on LB agar plates supplemented with 50 $\mu g \cdot mL^{-1}$ kanamycin (Km). 50 µL of overnight grown culture (~16 hr) was transferred to SBM anaerobic media supplemented with 40 mM glycerol as the sole carbon and energy source and 40 mM fumarate as the electron acceptor. The first transfer was taken when the culture reached an optical density of ~0.3. A 50 µL aliquot was then taken and put into fresh anaerobic SBM containing 40 mM glycerol and 40 mM fumarate and allowed to grow for 24 hours. Culture transfers were repeated 10 times. After the last transfer, frozen glycerol stocks were made and the plasmid was extracted and conjugation experiments were done to transfer the plasmid to wildtype *S. oneidensis* MR-1. Conjugation in wildtype MR-1 was performed to confirm that mutations occurred on the plasmid and not on the *S. oneidensis* genome. The extracted plasmid DNA was used for sequencing of the anaerobically evolved plasmid (pGUT2PET_A).

Sequencing pGUT2 and pGUT2PET plasmids and annotation

Plasmid DNA was extracted from strains JG993 (pGUT2), JG2069 (pGUT2_A), JG1327 (pGUT2PET), JG1083 (pGUT2PET_O) and JG1083_A (pGUT2PET_A) using the Wizard miniprep kit from Promega. A reference genomic DNA (*G. sulfurreducens*) for ease of assembly along with all the barcoded plasmids was sent for Illumina MiSeq V2

sequencing at the University of Minnesota Genomics Centre. Nextera XT method was used for library preparation and paired-end reads, 250 cycles were used for an Illumina MiSeq sequencing run. Bre-seq pipeline and SPADES assembler were used to assemble and compare sequence with the reference sequence. Prokka software was used to annotate the genes. Once annotated, the mother plasmid sequence was then used to compare sequences from the evolved plasmids to determine the route of evolution in the plasmid sequence.

pGUT2 plasmid re-construction

Primers used are listed in Table 4.2. To clone *glpFK* back to pGUT2 to repair the mutations, primer pairs that were used initially to clone glpFK from E. coli were used but the cloning experiment failed. *G. subterraneus* is a gram negative Fe(III)-reducing bacterium that has the ability to utilize glycerol as the sole carbon and energy source (Badalamenti et al, 2015). *G. subterraneus* genome also contains *glpFK* gene cluster. To re-construct the pGUT2 plasmid, we chose glpFK from *G. subterraneus*. *G. subterraneus* genomic DNA was used as PCR template with primers KJ3 and KJ5. Amplified product was run in agarose gel to check the size of amplified product. QIAquick PCR purification kit was used to purify the amplified product and then digested with XbaI and PsiI, cleaned again. pGUT2 was digested with XbaI and PsiI, phosphatase treated and run on agarose gel and the larger fragment was then excised from gel and purified. Purified PCR product was then cloned into the digested and phosphatased pGUT2 making pGUT2_Geo. The same procedure was used to clone *glpFK* from *E. coli* to pGUT2.

Transformation and conjugation procedures were performed to transfer pGUT2_F to wildtype *S. oneidensis* MR-1.

Primer	Restriction Site	Sequence
Primers for *glpFK* amplification from *G. subterraneus*:		
KJ3	XbaI	GC TCTAGA GCGGCTCTCCGCTAAGGATTGAAG
KJ5	PsiI	CACGC TTATAA CCAACCAATGGAACAGTCAGCGG
Primers for *glpFK* amplification from *E. coli* K12:		
KJ7	NotI	AG GCGGCCGC GAGGGATTATTGATGTGTGCGGG
KJ8	XbaI	GC TCTAGA GATGGAATAAATGGCGCGATAACG
Primers for sequence verification of *glpFK* from *G. subterraneus*:		
KJ11 FP-1		GATTTCAATCTGGCCTGCTC
KJ12 RP-1		GATTGATAGCGTAGCCGGT
KJ13 FP-2		AACCTGGTGACGGAGATCATCG
KJ14 RP-2		CAGACGATAGCGTTGCAGTAG
KJ15 FP-3		AGCACCCGCTGTATGATC
KJ16 RP-3		GTAGCAGACAGTGGTCACCA
KJ17 FP-4		AAATACTTACGGCACCGGCTG
KJ18 RP-4		CCAACCAATGGAACAGTCAGCGG

KJ19 FP-5	CGGTGATTCGTCCCAAAGTC
KJ20 RP-5	GATGTGGCTGCCTTCAGCTT

Table 4.2 Primers used to clone *glpFK* genes and reconstruct pGUT2 plasmid and primers for sequence verification of the genes replaced.

Growth on glycerol

Strains were grown overnight aerobically in LB media supplemented with 50 $\mu g \cdot ml^{-1}$ Km from single colonies of freshly streaked LB agar plate containing 50 $\mu g \cdot ml^{-1}$ Km. 50 µL of this culture was inoculated into LB anaerobic media containing 40 mM glycerol, 40 mM fumarate and 50 $\mu g \cdot ml^{-1}$ Km and SBM anaerobic media supplemented with 40 mM glycerol, 40 mM fumarate and 50 $\mu g \cdot ml^{-1}$ Km to an optical density of ~0.04. The culture tubes were incubated at 30 °C in a shaker. Periodically, OD at 600 nm wavelength were taken and 0.3 mL aliquots were taken by sterile anaerobic syringe and needle and samples were frozen at -20 °C for HPLC analysis.

HPLC analysis

Metabolites were quantified by high performance liquid chromatography (HPLC) on an Aminex 87H column using UV-Visible detector set at 210 nm and refractive index detector. The system (Shimadzu Scientific) consisted of an SCL-10A system controller, LC-10ATvp Liquid chromatograph, SIL-10AF autoinjector, CTO-10A column oven, RID-10A refractive index detector and SPD-10A UV-Vis detector. The separation of compounds was performed with an Aminex HPx-87H guard column (Bio-Rad) and

Aminex HPX-87H cation exchange column (Bio-Rad). The mobile phase was 0.015N H2SO4 with a flow rate of 0.4 ml/min. The column was maintained at 46°C and the injection volume was 50 µL.

Fe(III)-citrate reduction assay

To test the ability of S. oneidensis containing pGUT2_Geo plasmid to grow on Fe(III)-citrate as the electron acceptor and glycerol as the sole electron donor. Wildtype *S. oneidensis* MR-1 was used as negative control. Strains from overnight aerobically growing LB culture supplemented with 50 $\mu g \cdot ml^{-1}$ Km were transferred to minimal medium containing 15 mM lactate or 10 mM glycerol as the electron donor and 55 mM Fe(III)-citrate as electron acceptor. Samples were collected periodically and diluted 10-fold in 0.5 N HCl. Fe(II) production over time was measured by taking 50 µL of 0.5 N HCl diluted samples and analyzed in microtiter well plate with 300 µL, 2g/L Ferrozine reagent in 100 mM HEPES buffer, followed by absorbance measurement at 562 nm (Lovley and Philips, 1987). Real-time Fe(III) citrate reduction experiments were carried out in 96-well plates as previously described with the following modifications (Chan et al, 2015). Briefly, strains cultured for 12 hours in anoxic SBM containing 20 mM lactate or 40 mM glycerol and 50 mM fumarate were washed and re-suspended in SBM to a final optical density measured at 600 nm (OD600) of ~0.1. Cells were added to 96-well plates followed by a 10:1 volume of buffered solution containing sodium DL-lactate (10 mM), Fe(III) citrate (5 mM), NaCl (50 mM), K_2HPO_4 (1.3 mM), KH_2PO_4 (1.7 mM), $(NH_4)SO_4$ (1.7 mM), $MgSO_4$ (0.5 mM), HEPES (100 mM), and Ferrozine reagent (2g/L).

All preparations and incubations were carried out in an OMNI-LAB glove box (Vacuum Atmospheres Company) under a nitrogen atmosphere. Fe(II) concentration was assayed at 1 minute intervals at 625 nm using a SpectraMax M2 multimode microplate reader (Molecular Devices) over four hours. Fe(II) concentration was calculated using a standard curve generated with ferrous sulfate dissolved in 0.5 N hydrochloric acid.

Bioreactor setup

The bioreactor consists of a graphite electrode measuring 0.5 cm x 3 cm x 1 mm attached to a platinum wire loop serving as the working electrode. A platinum wire attached to a glass tubing soldered with silver solder and copper wire for electrical connection serves as the counter electrode. Ag/AgCl reference electrodes were connected to bioreactors via a salt bridge assembled from glass tubing and a nanoporous Vycor frit (BioAnalytical Systems, West Lafayette, IN). Salt bridge was filled with 0.1 M KCl in 1% agarose and topped with 3M KCl. All the electrodes were fitted into a PEEK (a polyether ether ketone thermoplastic polymer) top placed onto 25 mL glass cone (Bioanalytical Systems, IN). The graphite working electrode was polished with 400 grit sandpaper, rinsed in 1 N HCl and cleaned by sonicating in deionized water twice for 10 min. The polished graphite electrode was then attached to a platinum wire loop using nylon bolt and nut (McMaster Carr). The bioreactors were monitored and potentials were maintained using a 16-channel VMP® potentiostat (Bio-Logic SA). Reactors were continuously flushed with humidified argon gas to maintain anoxic conditions, and a

temperature of 30°C was maintained by using a circulating water bath. Reactors were stirred continuously using stir bars.

Electrochemical techniques

Electrochemical techniques were used to monitor the current production and biofilm formation of *S. oneidensis* MR-1 in modified bioreactors. Chronoamperometry (CA) measures current production over time where the working electrode is poised at a set potential versus the reference electrode. The working electrode was poised at 0.240 V vs SHE (Standard Hydrogen Electrode). Bioreactors containing 14 mL SBM growth medium with 40 mM glycerol and 40 mM fumaratee were inoculated with 1 mL of mid-exponential *S. oneidensis* culture (~0.5 – 0.6 OD) grown in LB anaerobic medium containing 20 mM lactate or 40 mM glycerol and 40 mM fumarate, and incubated for 96-100 hours. After 96 hours, cyclic voltammetry (CV) was conducted over a potential range from -0.555 V vs SHE to +0.450 V vs SHE at a scan rate of 1mV/sec.

Results and Discussion

All plasmids have SNP in *glpF* gene

Sequence analysis of pGUT2 (JG993), pGUT2_A (JG2069), pGUT2PET (JG1327), pGUT2PET_O (JG1083), and pGUT2PET_A plasmids showed a single nucleotide deletion at 146th position in *glpF* gene on all the plasmids causing a frameshift mutation. This mutation was found in all the versions of the plasmid. Frame shift caused modification in reading frame causing transcription termination due to presence of a new

stop codon after 146th nucleotide. Another start site was found overlapping with first reading frame indicating formation of two incomplete transcripts, suggesting GlpF does not function properly when strains were tested for growth in glycerol. pGUT2_A plasmid was found to have a single nucleotide deletion in the *glpK* gene. The size of pGUT2 original plasmid was found to be 10,205 bp and that of pGUT2_A acclimatized plasmid was 10,204 bp. Evolution route of pGUT2 plasmids are shown in figure 4.3.

Plasmid	**Size (bp)**	**Mutations found**
pGUT2	10,205	single base deletion in *glpF*
pGUT2_A	10,204	single base deletion in glpF, single base deletion in *glpK*. (evolved from pGUT2_A)
pGUT2PET	13,452	single base deletion in *glpF*.
pGUT2PET_O	13,297	single base deletion in *glpF*, 154 bp deletion in the intergenic region of *glpK* and *tpiA*, including few last nucleotides from *glpK*. (evolved from pGUT2PET)
pGUT2PET_A	13,836	single base deletion in *glpF*, 154 bp deletion in the intergenic region of *glpK* and *tpiA* including few last nucleotides from *glpK*. *pdc*, *adh*, *kan*^{R} genes were replaced with some DNA from the *S. oneidensis* megaplasmid.

(evolved from pGUT2PET_O)

Table 4.3 Summary of sequencing result for all the plasmids.

From sequence analysis, we found that size of pGUT2PET original plasmid to be 13,452 bp where as pGUT2PET_E+O2 (evolved aerobically) plasmid to be 13,297 bp and pGUT2PET_E-O2 (evolved anaerobically) to be 13,836 bp. When pGUT2PET original plasmid was acclimatized aerobically to grow in glycerol, sequence analysis showed 154 bp deletion in the acclimatized version of pGUT2PET plasmid. The deletion event occurred towards the end of *glpK* gene and before *tpiA* gene in the intergenic region including some of the last nucleotides of *glpK*. Same 154 bp deletion was detected in the anaerobically acclimatized version of pGUT2PET plasmid (pGUT2PET_A) (Table 4.3).

Changes in *glpK* gene are thought to compensate for the frame shift mutation in *glpF* gene. It is reported that GlpK physically interacts with the C-terminal end of GlpF and activates it (Voegele et al., 1993). Fu et al. (2000) provided structure of glycerol facilitator and the functioning of this channel. 146th nucleotide deletion disrupts the selectivity channel and truncates one of the periplasmic helices, impairing the protein function (Figure 4.2).

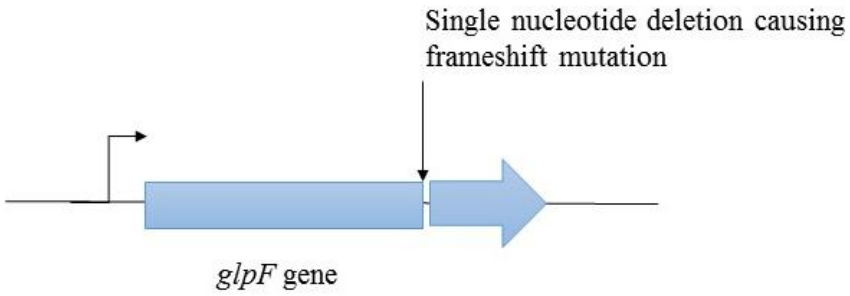


Figure 4.2 SNP in *glpF* gene causing frameshift mutation in all the plasmids.

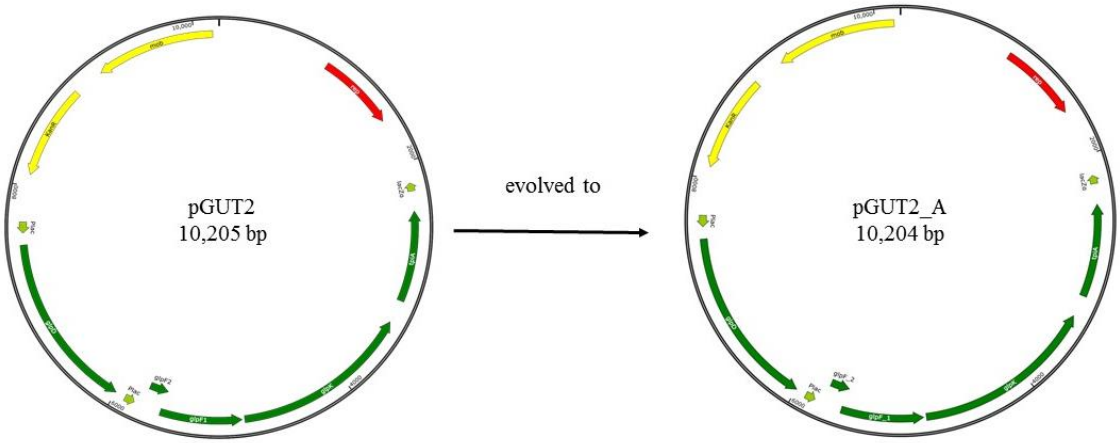


Figure 4.3 Evolution route of pGUT2 plasmid.

pGUT2PET_A plasmid showed the most mutations. (Figure 4.4) When pGUT2PET plasmid was adapted anaerobically to grow on glycerol, sequence analysis showed occurrence of a recombination event between the *pdc*, *adh and* kan^R genes of the plasmid and a portion of DNA of megaplasmid of *S. oneidensis*. The plasmid still conferred kanamycin resistance ability without the presence of kan^R gene. The recombination event caused nucleotidyl transferase gene, a transposase gene and a gene

coding for hypothetical protein to be incorporated to the pGUT2PET_A plasmid in place of *pdc*, *adh* and kan^R gene. We found out that the nuclotidyl transferase gene is responsible for conferring kanamycin resistance to the plasmid as the kanamycin sensitive strains containing pGUT2PET_A plasmid conferred Km resistance to the strains when grown on LB medium containing 50 $\mu g \cdot mL^{-1}$ Km.

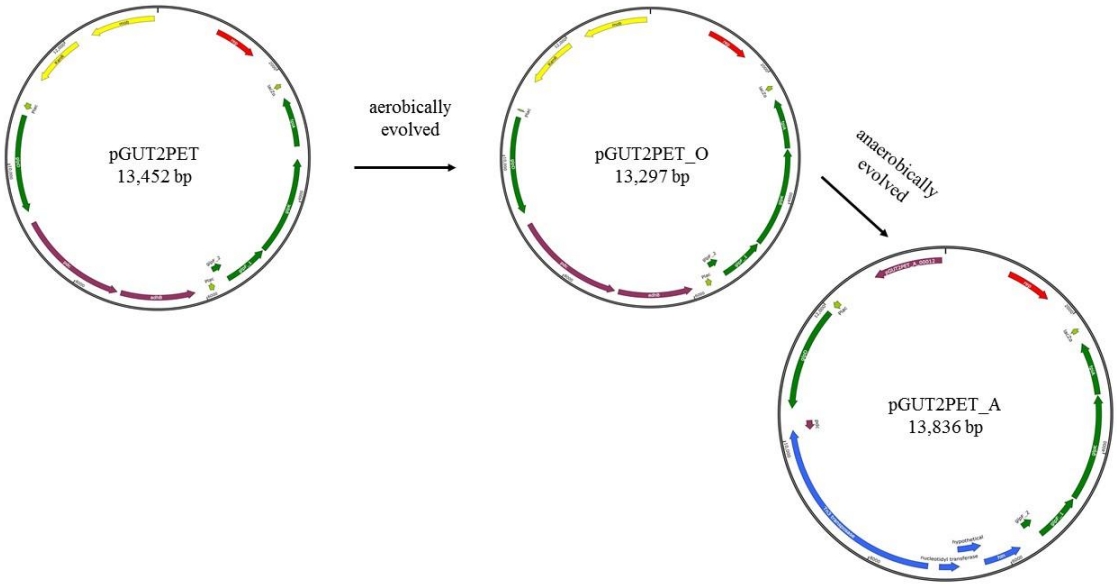


Figure 4.4 Evolution route of pGUT2PET plasmids.

In order to repair the single nucleotide deletion in the *glpF* gene, we chose to reconstruct the plasmid by replacing *glpFK* genes from the plasmid with the *glpFK* genes from *E. coli* K-12 again, but the cloning wasn't successful. So, we chose to clone *glpFK* genes from *G. subterraneus* which was successful. Figure 4.5 shows the growth curve of MR1 containing pGUT2_F plasmid in anaerobic SBM containing glycerol and fumarate.

The doubling time of MR1 with pGUT2_F plasmid anaerobically in glycerol was measured to be 4.25 ± 0.8 hours.

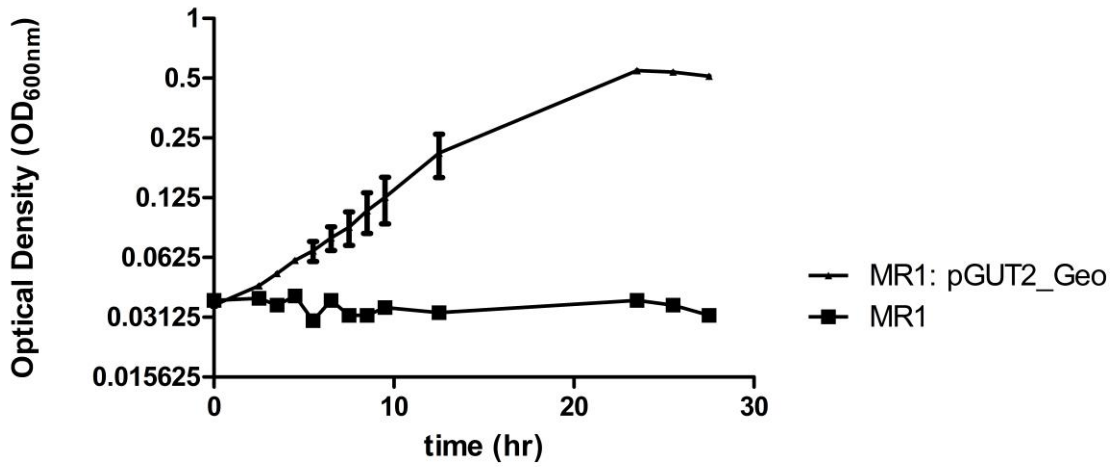


Figure 4.5 MR-1 containing pGUT2_Geo plasmid grown anaerobically with 40 mM glycerol as the sole carbon source and 40 mM fumarate as sole electron acceptor. WT MR-1 was used as negative control.

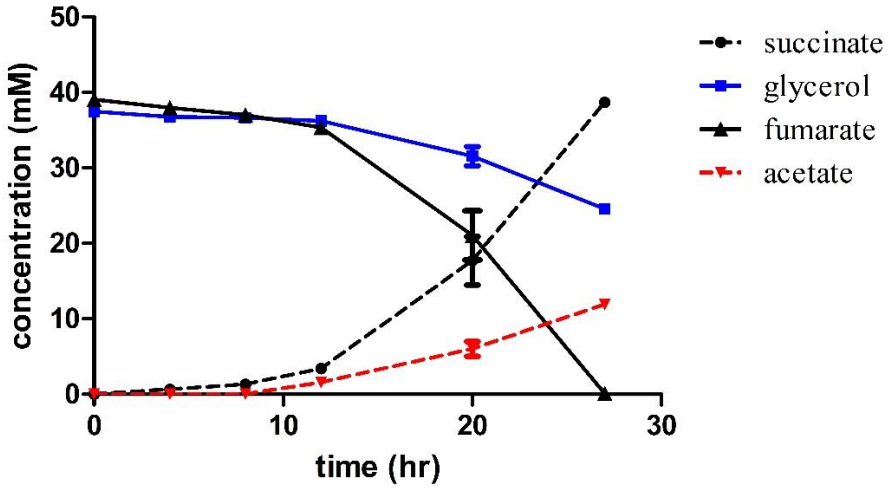


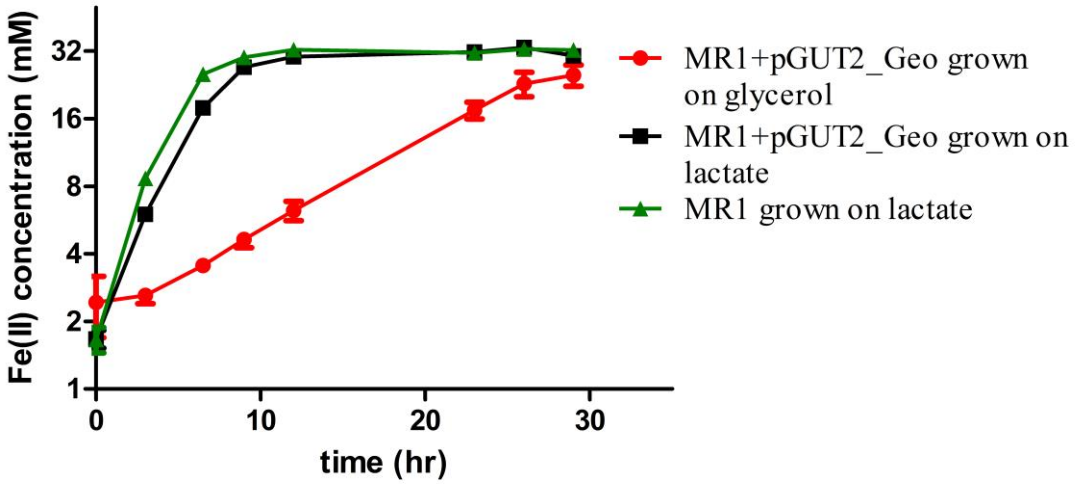
Figure 4.6 HPLC data for MR-1 + pGUT2_Geo growing anaerobically in glycerol as the electron donor and fumarate as the acceptor. Glycerol was oxidized to acetate and fumarate was reduced to succinate.

HPLC analysis was done on *S. oneidensis* cells containing pGUT2_Geo plasmid (Figure 4.6). The metabolites concentration was found to be in stoichiometric ratios. No further acclimatization of plasmid was done.

To check the Fe(III) citrate reduction ability of MR-1 with pGUT2_Geo in presence of glycerol, Fe(III)-citrate growth assay was performed to check the growth of MR-1 containing pGUT2_Geo plasmid on Fe(III)-citrate medium containing 40 mM glycerol as the sole electron donor. MR-1 cells with pGUT2_Geo plasmid were pre-grown on anaerobic LB medium containing 40 mM glycerol and 40 mM fumarate and inoculated at mid-exponential phase (~0.2- 0.3 OD) to two different tubes: i) SBM containing 40 mM glycerol as electron donor and 55 mM Fe(III)-citrate as the electron acceptor, ii) SBM containing 20 mM lactate as electron donor and 55 mM Fe(III)-citrate

as the electron acceptor, supplemented with 50 $\mu g \cdot mL^{-1}$ Km to maintain the selective pressure for plasmid. Wildtype MR-1 cells was inoculated from LB anaerobic medium containing 20 mM lactate and 40 mM fumarate to SBM containing 20 mM lactate and 55 mM Fe(III)-citrate. Wildtype MR-1 was used as a positive control. The growth assay suggests, slower growth in Fe(III)-citrate respiration when cells containing pGUT2_Geo are grown in presence of glycerol compared to Fe(III)-citrate respiration rates when cells containing pGUT2_Geo were grown with lactate as the electron donor (Figure 4.7 A). Resting cell real time Fe(III)-citrate reduction assay was also done to measure Fe(III) reduction rates in non-growing cells. Results show that Fe(III) reduction capability is disrupted in the cells with glycerol as the sole electron donor (Figure 4.7.B).

A.



B.

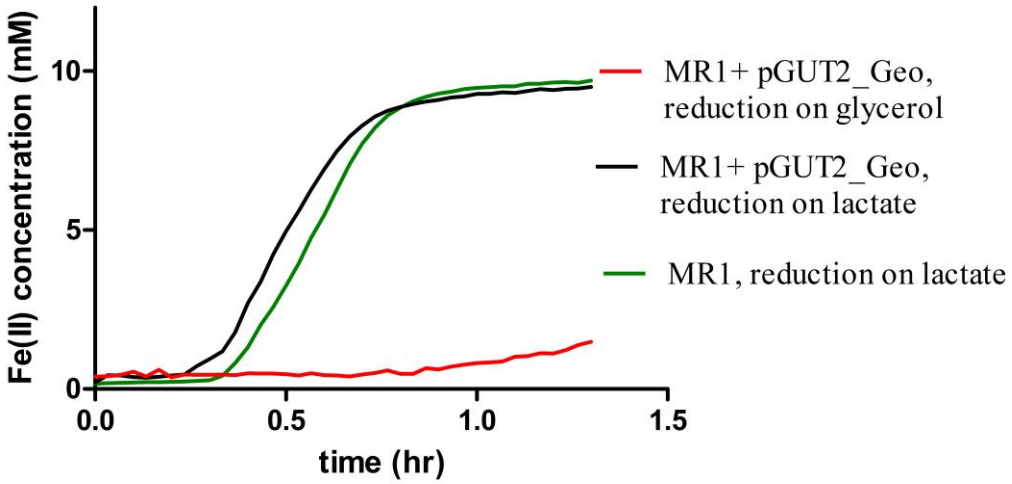


Figure 4.7 Reduction of Fe(III) to Fe(II) in MR1+ pGUT2_Geo with lactate or glycerol as the sole electron donor and wildtype *S. oneidensis* MR-1 with lactate as electron donor. A: Fe(III)-citrate growth over time with the growth of cells. B: Real time Fe(III)-citrate reduction of resting cells showing inability to reduce Fe(III) in MR1 + pGUT2_Geo when glycerol was the sole electron donor.

Similar results were observed when MR-1 containing pGUT2_Geo cells were grown in bioreactors with poised electrode as the electron acceptor. Cells were able to

respire fumarate present in the SBM growth media in presence of glycerol but were unable to respire electrodes (Figure 4.8). Initial phase of cell attachment can be seen when all the fumarate is reduced but cells never respired poised electrodes. Cells containing pGUT2_Geo plasmid were pre-grown in LB anaerobic medium containing i) 20 mM lactate, 40 mM fumarate and 50 $\mu g \cdot mL^{-1}$ Km, ii) 40 mM glycerol and 40 mM fumarate and 50 $\mu g \cdot mL^{-1}$ Km. At mid-exponential growth phase, 1 ml of MR-1: pGUT2_Geo cells were inoculated into bioreactors containing 40 mM glycerol and 40 mM fumarate. After inoculation, current was measured over time (Figure 4.8)

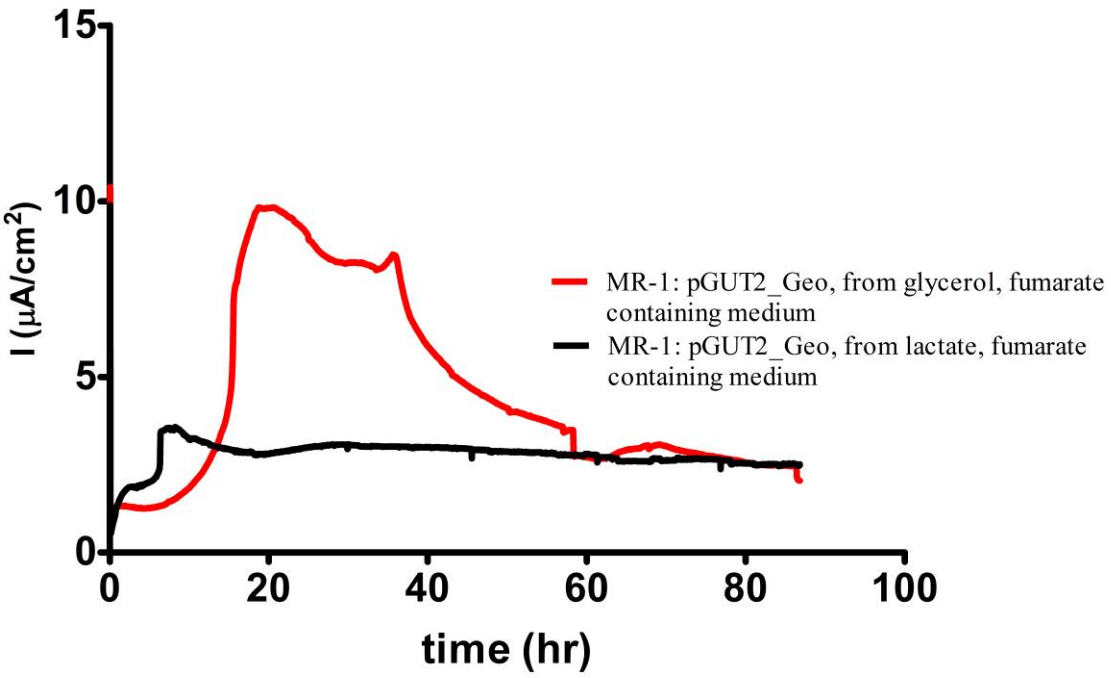


Figure 4.8 Growth of MR-1 containing pGUT2_Geo plasmid in bioreactors set up to respire electrodes set at a poised potential of +0.24 V. Cells were grown in lactate or glycerol first and then transferred to bioreactors containing SBM with 40 mM glycerol and 40 mM fumarate.

MR-1 cells containing pGUT2_Geo plasmid when grown with Fe(III)-citrate or electrode as the electron acceptor with glycerol as the sole electron donor, shows the inability of these cells to respire Fe(III)-citrate or electrode suggesting glycerol oxidation when coupled to electrode reduction or Fe(III)-citrate reduction dirupts the transfer of electrons via Mtr pathway in *S. oneidensis*. Mtr pathway is required for S. oneidensis to reduce electrodes. The mechanism behind the slow turn over of glycerol in *S. oneidensis* is still unknown.

From all the experiments, it was found that *S. oneidensis* MR1 containing reconstructed plasmid (pGUT2_Geo) grows anaerobically when glycerol oxidation is coupled to fumarate respiration but does not oxidize glycerol faster when coupled with Fe(III) or poised electrode respiration. The results suggests some evolution might be needed for pGUT2_Geo plasmid to be able to respire Fe(III)-citrate or electrodes.

Chapter 5

Conclusions and Future directions

Electrochemically active bacteria require a controlled anaerobic environment for their culture in bioelectrochemical systems. New designs using a PEEK material top and separate inlet and outlet ports provided a better sealed system that can be modified easily. Experiments were performed with *S. oneidensis* only, however other dissimilatory metal reducing bacteria can be utilized for measuring efficiency in the new reactors. New reactors could also be used to isolate and culture other electrochemically active bacteria from subsurface environments. Modification in the design will be needed to scale up the bioelectrochemical system for industrial processing.

One modification allowed us to study hydrogen metabolism in *S. oneidensis* biofilms in single chambered reactors. Hydrogen recycling contributed about 40% of the current production in single chambered bioreactors with *S. oneidensis*. Deletion of hydrogenase genes increased the efflux of electrons to the poised electrode and this can be used as a powerful tool for improving the efficiency of microbial fuel cells. The hydrogen formed at the cathode could also be recovered and stored. The modified reactor design could also be helpful for studying interspecies electron transfer by eliminating the hydrogen into the media.

Adaptation route of pGUT2 plasmids for the growth of *S. oneidensis* in glycerol was elucidated and a single nucleotide deletion in the *glpF* gene was found in all versions of the plasmid. MR-1 with a new reconstructed plasmid (pGUT2_Geo) grew at rates

comparable to MR-1 with adapted versions of the plasmid (pGUT2_A) under anaerobic conditions with glycerol as the sole carbon and energy source and fumarate as the electron acceptor. The mechanism behind slow growth when glycerol oxidation is coupled to metal reduction (Fe(III)-citrate) is still unknown. Overall, sequence of all pGUT2 (pGUT2_O, pGUT2_A) and pGUT2PET (pGUT2PET, pGUT2PET_O, pGUT2PET_A) plasmids was elucidated and evolution route directing mutations was figured. A new plasmid containing glpFK gene cluster from *G. subterraneus* was cloned into pGUT2 plasmid lacking *glpFK*.

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